Elucidating the role of reduced Gfrα1-receptor expression *in vivo* and its connection to Hirschsprung's disease and associated enterocolitis

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Tiivistelmä/Referat – Abstract

Kirjallisuuskatsaus: Enteerinen hermosto (ENS) pystyy säätelemään suoliston toimintaa keskushermostosta riippumatta, jonka takia enteeristä hermostoa onkin kutsuttu ”toisiksi aivoiksi”. Enteerinen hermosto kehittyy sikiön kehityksen aikana muodostuvasta enteerisestä hermostopienasta (enteric neural crest derived cells, ENCCs) ja sen kehittyminen on monimutkainen prosessi, jonka säätelyyn liittyi useita eri signaalireittejä. GDNF/Gfrα1/RET signaalireitti on erityisen tärkeässä osassa enteerisen hermoston kehityksen kannalta, sillä se säätelee enteerisestä hermostopienasta syntyvien hermosolujen selviytymistä, lisääntymistä, migraatiota ja erilaistumista.

Hirschsprungin tauti on yleisin suolen motiliteettiin vaikuttava synnynnäinen sairaus. Hirschsprungin tauti johtuu enteerisen hermoston puuttumisesta paksusuolen distaalisessa osassa ja sen esiintyvyys on 1:5000. Suolen motiliteetin häiriintymisestä johtuen Hirschsprungin taudista kärsivillä vastasyntyneillä esiintyy ummetusta, oksentelua, vatsakipuja, vatsan turvotusta ja joissain tapauksissa ripulia. Taudin vakavin, hengenvaarallinen oire on Hirschsprungin tautiin liittyvä enterokoliitti, jota esiintyy 30-50% potilaista. Hirschsprungin tautia hoidetaan leikkaamalla suolen osa, josta enteerinen hermosto puuttuu, mutta enterokoliitin riski on onnistuneenkin leikkauksen jälkeen suuri. Histologisia näytteitä analysoimalla on saatu selville, että enterokoliittiin liittyy useita muutoksia suolen epiteelissä kuten pikarisolujen hyperplasiaa, muutoksia musiiniprofiilissa, musiinin retentiota, epiteeliin rakenteen vaurioitumista, inflammaatiota ja bakteerien kiinnittymistä epiteeliin. Muutoksien tarkan järjestyksen määrittämistä on kuitenkin osittain rajoittanut sopivan eläinmallin puute.

Hirschsprungin taudin suurin riskitekijä on mutaatio *RET*-geenissä, ja se esiintyy noin puolella Hirschsprungin tautia sairastavalta. RET on reseptori solukalvolla, jonka kautta muun muassa GDNF/Gfrα1/RET signaalireittiin vaikutukset välittyvät. Poistogeenisille *Ret*-, *Gfra1*- tai *Gdnf*-hiirille ei kehity munuaisia, jonka takia poikaset kuolevat vastasyntyneinä. Tämän takia Hirschsprungin tautiin liittyvää hiirimallia, joka vaikuttaa GDNF/Gfrα1/RET signaalireittiin ei ole tällä hetkellä vielä olemassa.

Kokeellinen osa: Jaan-Olle Andressoon luoma GFRa1 hypomorfinen hiiri (*Gfra1*$^{hypo/hypo}$) on ensimmäinen GDNF/Gfrα1/RET signaalireittiin vaikuttava hiirimalli, jolla voidaan tutkia Hirschsprungin tautia syntymän jälkeen. Näiden hiirien *Gfra1*-geeniin ilmentyminen on vähentynyt 70-80% kehittyvässä suolessa ja munuaisissa, jonka seurauksesta distaalisesta paksusuolesta puuttuu enteerinen hermosto Hirschsprungin taudille tyypilliseen tapaan, mutta munuaisten kehitys ei ole häiriintynyt. *Gfra1*$^{hypo/hypo}$ hiiret lopetetaan hyvinvointi ongelmista johtuen P7-P25, jonka aikana on mahdollista tutkia enterokoliitin syntymekanismeja.

Gfra1$^{hypo/hypo}$ hiirien histologinen analysointi osoitti, että hiirillä esiintyy pikarisolujen hyperplasiaa ja musiiniprofiili oli muuttunut happamasta musiinista neutraalimmaksi musiiniksi distaalisessa paksusuolessa. Pikarisolujen hyperplasia havaittiin P10, mutta musiiniprofiilin muutos pystyttiin havaitsemaan jo P5. Tarkkaa tietoa siitä minkä takia minkä takia musiiniprofiilin muutos tapahtuu ei ole, mutta näyttää siltä, että se edeltäisi pikarisolujen hyperplasiaa. qPCR-analyysin avulla selvisi, että *Muc2*-geenin mRNA-tasot olivat kohonneet P5 ja P10. *Muc2* on yleisin suoleen eritettävä musiini, ja se suojaa suolen epiteeliä suolen luumenissa olevilta patogeeneiltä. Sen lisäksi *Tnfa*-geenin ilmentyminen oli koholla P10. Edellä mainittuja muutoksia ei havaittu ohutsuolessa, jossa *Gfra1*-geenin ilmentyminen on vähentynyt, mutta enteerinen hermosto on kehittynyt normaalisti. Onkin siis todennäköistä, että distaalisessa paksusuolessa tapahtuneet muutokset ovat seurausta enteerisen hermoston puuttumisesta eikä GDNF/Gfrα1/RET signaalireitin heikentyneestä toiminnasta. Seerumin analysointi osoitti, että systeemisestä inflammaatiosta ei ollut merkkejä P10-P14-P16, vaikkakin yhdellä *Gfra1*$^{hypo/hypo}$ hiirellä oli korkeat IL6 ja TNFa tasot P14-P16. Näyttääkin siltä, että inflammaatio ei ole enterokoliitin alkuvaiheen tapahtumia ja pikarisoluissa tapahtuvat muutokset edeltävät inflammaatiota.

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HSCR, HAEC, ENS, ENCC, GFRα1, GDNF, MUC2

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Tiivistelmä/Referat – Abstract

Literature review part: The enteric nervous system (ENS) often called "the second brain" is considered its own autonomic division that can independently regulate gut function. The ENS is derived from enteric neural crest-derived cells (ENCCs), which colonize the gut during development. Development of the ENS is a complex process, and many signalling pathways are required for a properly functioning ENS, especially GDNF/Gfrα1/RET signalling controlling survival, proliferation, migration, and differentiation of ENCCs.

Hirschsprung's disease (HSCR) is the most common congenital disease affecting gut motility. The prevalence of HSCR is 1:5000, and it is characterized by a complete lack of enteric neurons (aganglionosis) in the distal colon. Due to impaired intestinal motility, infants may have constipation, emesis, abdominal pain or distention, and, in some cases, diarrhea. The most life-threatening symptom is HSCR-associated enterocolitis (HAEC), which occurs in 30-50% of patients. Routine treatment for HSCR is a surgical operation called "pull through" in which the aganglionic segment is removed, and the remaining ganglionic segment is joined to the anus. However, the risk of developing HAEC after successful surgery still exists. Histopathological analysis has revealed that HAEC is accompanied by various changes in the gut epithelium, especially in mucin-producing goblet cells. These changes include hyperplasia of the goblet cells, altered mucin profile, retention of mucin, damaged and disorganized epithelium structure, inflammation, and bacterial adherence to the epithelium. However, a lack of suitable postnatal HSCR mouse models has partially hindered the progress of pinpointing the exact order of these events.

A RET mutation found in half of the patients is overwhelmingly the biggest risk factor for HSCR. RET is a receptor on the cell membrane that mediates the effects in GDNF/Gfrα1/RET signaling pathway. of Knock-out mice of Gdnf, Gfra1 and Ret all have intestinal aganglionosis, resembling HSCR. However, to date, no mouse models of HSCR affecting GDNF/Gfrα1/RET signalling exist because pups are born without kidneys and die soon after birth.

Experimental part: The GFRa1 hypomorphic mouse line (Gfra1hypo/hypo) created by Dr. Jaan-Olle Andressoo is the first successful model that survives past birth while manipulating GDNF-Gfrα1-RET signalling and phenocopying HSCR. These mice have 70-80% reduction in the expression of Gfrα1 in the developing gut and kidneys, which is sufficient to cause aganglionosis in the distal colon, yet not enough to impair kidney development.These mice are sacrificed between P7-P25 because of welfare problems yet giving a time window for analysis of the development of HAEC.

Histological analyses revealed that Gfra1hypo/hypo mice had goblet cell hyperplasia and a shift away from acidic mucin production in the distal colon. Goblet cell hyperplasia was first observed at P10, but the shift in mucin profile already appeared at P5. It is not known what causes goblet cells to change their mucin production, but it seems to be the earliest histopathological change in HAEC preceding goblet cell hyperplasia. qPCR-analysis revealed that Muc2, the main secreted mucin that protects epithelium from invading pathogens, was upregulated at both P5 and P10. mRNA levels of Tnfa were also upregulated at P10. The aforementioned changes were not observed in the duodenum where the ENS had developed normally despite the reduction in Gfra1 expression. This indicates that the changes observed in the colon are likely due to the lack of ENS innervation, rather than a direct effect from GDNF-GFRa1-RET signalling itself. Finally, serum analysis indicated that systemic inflammation did not occur from P10-P16, although one Gfra1hypo/hypo animal had high levels of IL6 and TNFa at P14-16. This indicates that inflammation is not an early stage event and it is preceded by goblet cells related changes. In conclusion, changes in goblet cells seems to be earliest histopathological findings preceeding HAEC.

Avainsanat – Nyckelord – Keywords
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ABBREVIATIONS

AB	Alcian blue
cDNA	Complementary DNA
GDNF	Glial cell-derived neurotrophic factor
GFL	Glial cell line–derived neurotrophic factor family ligands
GFRα1	GDNF family receptor alpha-1
GIT	Gastrointestinal tract
GlcNAc6ST-2	N-acetylglucosamine 6-O-sulfotransferase-2
ENCC	Enteric neural crest derived cell
ENDRB	Endothelin receptor B
ENS	Enteric nervous system
HAEC	HSCR-associated enterocolitis
HSCR	Hirschsprung's disease
IL1β	Interleukin 1 beta
MAPK	Mitogen-activated protein kinase
P10	Post-natal day 10
PAS	Periodic Acid-Schiff base
PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative polymerase reaction
SPDEF	SAM pointed domain-containing Ets transcription factor
TGFβ1	Transforming growth factor beta 1
TNFα	Tumor necrosis factor alpha
WT	Wild type

I: LITERATURE OVERVIEW

1 INTRODUCTION

The enteric nervous system (ENS) is a network that consists of neurons and glial cells that innervate the gastrointestinal tract (GIT) (As reviewed in Furness et al. 2014). Unlike other peripheral organs, the GIT has extensive intrinsic innervation from the ENS. In humans, there are 200 - 600 million enteric neurons, more than all neurons in sympathetic and parasympathetic ganglia combined and equivalent to the number of neurons in the spinal cord. In resemblance to the central nervous system (CNS), the ENS has many phenotypically different neurons and most, if not all, of the same neurotransmitters (As reviewed in McConalogue and Furness 1994).

The ENS is considered its own autonomic division; the neurons in the ENS form circuits that regulate secretion, gut motility, hormone release, the movement of fluids across the epithelium, local blood flow, modification of nutrient handling, and communication with the immune and endocrine systems of the gut (As reviewed in Furness et al. 2014). In old textbooks, the autonomous nervous system (ANS) is depicted as efferent pathways that consist of preganglionic neurons and postganglionic neurons, and afferent sensory neurons that travel to the CNS through spinal and cranial nerve pathways. In the ENS, however, there are intestinofugal neurons that have their cell bodies in enteric ganglia, that project to the sympathetic ganglia, pancreas, gallbladder and trachea. The neuronal control of the GIT is a combination of interactions between local reflexes, reflexes that go through sympathetic ganglia, and reflexes that go through the CNS. In other words, the ENS is connected to the CNS, but it can also regulate gut functions independently, hence the term “the second brain.”

The ENS plays different roles in different part of the GIT (As reviewed in Furness et al. 2014). For example, neuronal control of the esophagus is largely controlled by the CNS. Even though the ENS has extensive innervation in the esophagus, its role is secondary to the CNS. The propulsion of the stomach is largely myogenic, but the CNS controls it. On the other hand, the ENS has more distinctive role in the small intestine and the colon (except for defecation). The CNS is connected to the ENS through the vagal, sympathetic and pelvic pathways. The vagus nerve innervates the distal small intestine

and the proximal colon to a lesser extent than the esophagus, the stomach and the proximal small intestine.

Hirschsprung's disease (HSCR) is a congenital disorder where the ENS is missing from the distal intestine at birth (As reviewed in Kenny et al. 2010). To understand its pathophysiology, knowledge about the structure of the gastrointestinal wall and how the ENS develops and is embedded is necessary. As we will discuss in more detail below, the GIT is comprised of functionally different layers and many different cell types (Figure 1).

The mucosa is the inner most layer in direct contact with the lumen (As reviewed in Yoo and Mazmanian 2017). The mucosa is composed of mucus, a single layer of epithelial cells, and the lamina propria. The mucus that is produced mostly by specialized cells in the epithelium called goblet cells covers the epithelium and protects it from invading pathogens. The single layer of epithelial cells in the gut is a heterogenous group of cells. In addition to goblet cells, the other common cell types are enterocytes, enteroendocrine cells, Paneth cells, and M cells. Enteroendocrine cells produce hormones when activated by various stimuli in the gut; these hormones can regulate gut functions such as motility and secretion. Paneth cells have a role in epithelial stem cell maintenance and they can also produce antibacterial compounds that protect host from invading pathogens. However, the most common cell type in the epithelium are enterocytes, primarily responsible for nutrient handling. Most of the nutrients are absorbed in the promixal parts of the gut where the epithelium is arranged in protruding tip-like structures called villi that maximize the surface area for absorption (As reviewed in Kiela and Ghishan 2016). Although water and NaCl are absorbed in the colon, there are no villi and minimal absorption of nutrients in the colon. Absorbed nutrients diffuse into the lamina propria, the prominently vascularized space underneath epithelial cells, and are transported to other locations of the body. The lamina propria is connective tissue that supports the structure of the epithelium, and it is home to many different types of immune cells that regulate the immune response of the GIT (As reviewed in Yoo and Mazmanian 2017).

Underneath the mucosa lies the submucosa separated by thin layer of smooth muscle cells (As reviewed in Yoo and Mazmanian 2017). In the submucosa, there are

submucosal neurons arranged in the plexus that regulate the secretion of the fluids and GI blood flow.

GI motility is generated by the muscularis propria, two distinct layers of smooth muscle cells (circular and longitudinal), and myenteric neurons that regulate their contraction (As reviewed in Yoo and Mazmanian 2017). The serosa, which is part of the membrane that lines the abnominal cavity lubricates the GI and is the outer most layer that is adjacent to the mesentery. Extrinsic fibers innervate the gut either directly or indirectly via enteric ganglia.

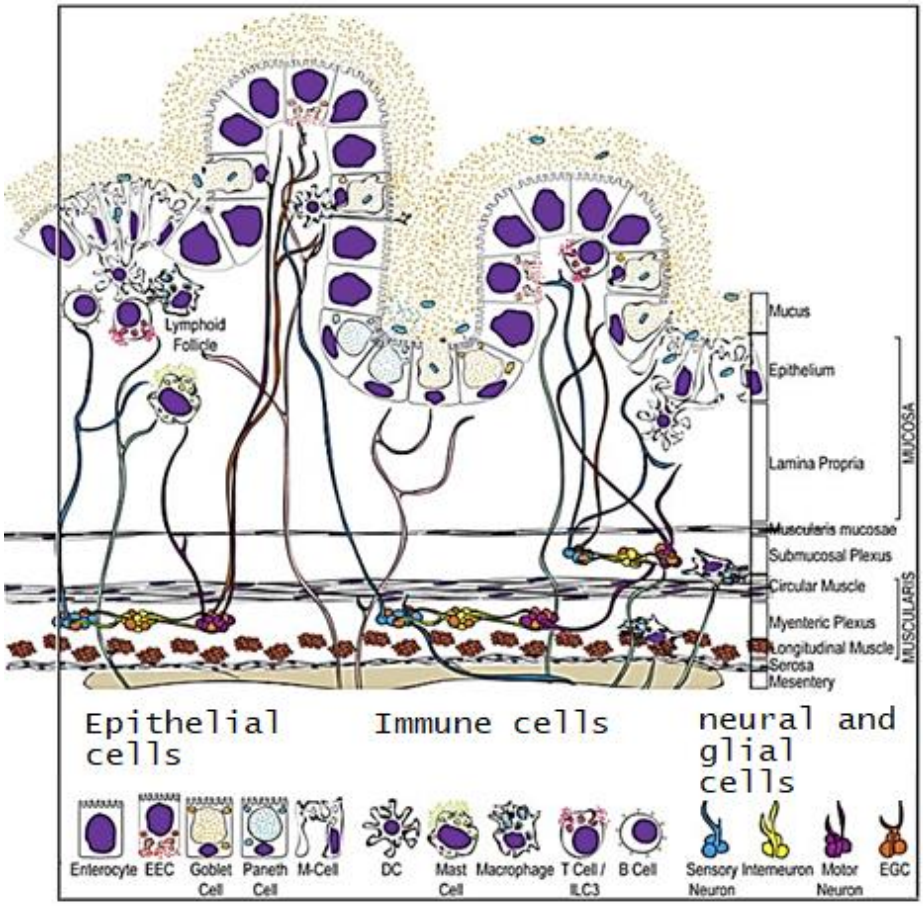


Figure 1. The structure of the GI wall. The GI wall consists of functionally different layers. The epithelium is mainly composed of enterocytes, but other cell types exist, such as, the enteroendocrine cells (EEC), the Paneth cells, the goblet cells and the M cells. The epithelium is covered by mucus which is primarily produced by goblet cells although other cell types can produce mucin. Enteric neurons are organized in two

distinct networks called the submucosal and myenteric plexus. Besides neurons, both plexuses have a great number of enteric glial cells that support these neurons and provide nutrients. Being exposed to number of bacteria, the gut is immunologically very active, and a number of different immune cells exist in the lamina propria and in the muscularis as depicted (Modified from Yoo and Mazmanian 2017).

The contraction of the smooth muscle tissue in the gut is driven by excitatory motor neurons that use acetylcholine (cholinergic motor neurons) as a primary neurotransmitter (As reviewed in Hansen 2003). Inhibitory motor neurons responsible for the relaxation of the smooth muscle tissue primarily use nitric oxide (nitrergic motor neurons). However, motoneurons co-express neuromodulators, such as neuropeptides, that also participate in the regulation of gut motility. Intrinsic primary sensory neurons (IPANs) form synapses with both excitatory and inhibitory enteric neurons directly or indirectly via interneurons (Figure 2). Among other stimuli, luminal distension and serotonin released from enterochromaffin cells (ECs), a special type of enteroendocrine cells storing around 95% of the total serotonin in the human body, can activate IPANs (As reviewed in Gershon and Tack 2007). Concurrent activation of both excitatory and inhibitory enteric neurons underlies the “peristaltic reflex” or “the law of the intestine” which was originally described by Bayliss and Starling in 1899 (Bayliss and Starling 1899). This is the movement of the content via oral contraction behind the bolus and anal relaxation in front of it. However, other researchers have had difficulties showing that local stimulation would lead to these previously theorized coupled contraction and relaxation patterns (Spencer et al. 1999).

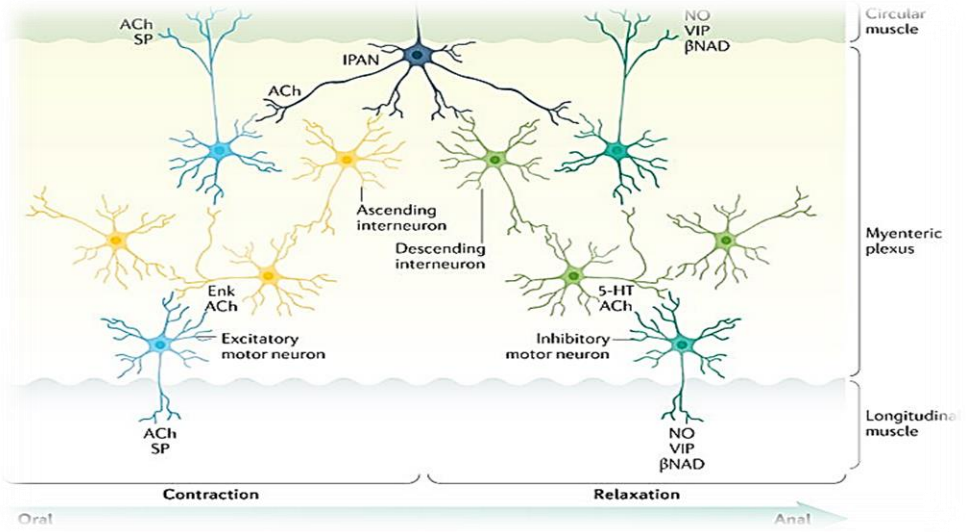


Figure 2. Peristaltic reflex. Intrinsic primary sensory neurons (IPANs) can be activated by luminal distension or by serotonin released from ECs. The contraction occurs when IPANs stimulate orally situated ascending interneurons or excitatory motor neurons directly. The contraction is mediated primarily by release of acetylcholine (ACh). Simultaneously, IPANs synapse with descending interneurons or directly with inhibitory motor neurons that cause the relaxation of anally situated muscle, mediated primarily by nitric oxide (NO), thereby reducing the intraluminal pressure (Modified from Rao and Gershon 2016).

2 Hirschsprung's disease (HSCR)

Hirschsprung's disease (HSCR) is the most common congenital disease affecting intestinal motility (As reviewed in Kenny et al. 2010). HSCR is characterized by a complete lack of enteric neurons (aganglionosis) typically in the distal colon. The lack of the ENS results in intestinal obstruction leading to a failure to thrive (Stewart and von Allmen 2003). While historically HSCR was fatal, nowadays babies suffering from HSCR have their aganglionic segment surgically removed.

HSCR can be divided into either short-segment or long-segment HSCR depending on the length of the aganglionic segment (As reviewed in Kenny et al. 2010). In short-

segment HSCR, which represents 80% of HSCR cases, the aganglionic segment is restricted to the most distal segment of colon called the rectosigmoid region. In long-segment HSCR, the aganglionic segment is more variable and may extend to the small intestine in some rare cases (As reviewed in Stewart & von Allmen 2003). The prevalence of HSCR is 1:5000, and there might be differences in ethnical populations (As reviewed in Kenny et al. 2010).

Mutations in *RET*, a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor (GDNF) family ligands, is overwhelmingly the biggest risk factor in HSCR; a coding mutation is found in 48% of familial cases and in 20% of sporadic cases (As reviewed in Heuckeroth 2018). Also, a non-coding *RET* variant in which a conserved enhancer-like sequence in intron 1 in *RET* is mutated increases the risk of HSCR significantly (Emison et al. 2005). The proper development of the enteric nervous system relies on molecular mechanism that govern survival, proliferation, migration, and differentiation of the enteric neuron precursor cells (As reviewed in Young and Newgreen 2001). RET-signalling plays an extremely important role in all those aspects. Therefore, factors that weaken RET-signalling increase the risk of developing HSCR. For example, *RET* heterozygosity increases the risk of HSCR by 3000-fold (As reviewed in Heuckeroth 2018). Transcription of *RET* is regulated by genes like *SOX10* and *PHOX2B*, and, indeed, 4% and 0.5% HSCR cases respectively have mutations in these genes. Mutations in endothelin receptor B (*ENDRB*), also important for ENS development, accounts for 5% of all HSCR cases and is the second most common genetic risk factor for HSCR. It should be noted that HSCR can be part of a syndrome like Trisomy 21 (Down syndrome) or Waardenburg syndrome. Indeed, 8% of HSCR patients have trisomy 21. In trisomy 21, the whole chromosome including the genes on chromosome 21, many of which are associated with HSCR, are triplicated, leading to an approximately 50% increase in gene dose. One of these genes is *collagen IV*, which can inhibit migration of neural crest cells at higher levels.

Interestingly, 80% of all HSCR patients are male. Sex-determining region Y protein found on the Y chromosome has a binding site on a *RET* regulatory element, and it competes with SOX10, an essential transcription factor in the ENS development. Animal studies have also shown that levels of END3 and ECE1, parts of endothelin

signalling, are found to be lower in male mice than in female mice, perhaps explaining part of the increased risk of developing HSCR in males.

Although HSCR has a superficially simple cause, the lack of ENS innervation, it has a complex genetic aetiology (As reviewed in Heuckeroth 2018). Even with modern clinical genetic testing, including exome sequencing, gene panels, and single nucleotide polymorphism (SNP) analysis, it is only possible to identify disease causing variants in half of the children that have HSCR leaving the other half of the HSCR cases unexplained. Currently, low-penetrance risk alleles combined with environmental factors during fetal development are thought to contribute to HSCR pathogenesis in cases where no known risk variants are found. Thus, diagnosis of HSCR poses a challenge.

2.1 Symptoms, diagnosis and treatment

Due to impaired intestinal motility, infants may have constipation, emesis, abdominal pain or distention, and, in some cases, diarrhea (As reviewed in Parisi 1993). Failure to pass meconium within the first 48 hours after the birth occurs in 50%-90% of newborns with HSCR. Most infants affected by HSCR have gut-related symptoms during the first two months of their life. In some cases, HSCR is diagnosed later in childhood or in even adulthood, and severe constipation lasting throughout life might indicate HSCR. The most life-threatening symptom is HSCR-associated enterocolitis (HAEC), which occurs approximately in 30% of the short segment cases and in 50% of the long segment cases (Frykman and Short 2012; Murphy and Puri 2005).

The definitive diagnosis of HSCR requires suction biopsy followed by histological analysis to show the absence of enteric ganglia (As reviewed in Parisi 1993). Supporting diagnostic tests include anorectal manometry, abdominal radiographs that can show the empty rectum and dilated proximal colon.

Routine treatment for HSCR is a surgical operation called “pull through” (As reviewed in Parisi 1993). In pull through, the aganglionic segment is resected, and the remaining

ganglionic segment is joined to the anus. Pull through operation is a highly successful operation, yet after surgery complications do occur; constipation and diarrhea are frequent, and HAEC is still common even after successful surgery (As reviewed in Heuckeroth 2018).

2.3 Hirscprung's disease associated enterocolits (HAEC)

Hirschsprung's disease associated enterocolitis or HAEC is the most fatal complication of HSCR and the most common cause of mortality and morbidity in neonates, is characterized by abdominal distension, fever, foul smelling stool, explosive diarrhea, and eventually sepsis and death if left untreated (As reviewed in Frykman and Short 2012; Pini Prato et al. 2011). HAEC can develop relatively suddenly, and its incidence seems to be unperturbed by surgical intervention both in humans and in animal models (Marty et al. 1995; Polley et al. 1985; Zhao et al. 2010). The aetiology of HAEC is poorly understood with several different, mutually non-exclusive theories proposed. Those theories can be roughly divided in three groups: impaired mucosal function, immune dysfunction and microbiome dysbiosis, reviewed in (As reviewed in Austin 2012), which I will briefly describe below.

2.3.1 Structure of the mucosa

The intestinal epithelium is covered by mucus (Johansson et al. 2008; as reviwed in Johansson and Hansson 2016). While the small intestine has a single, detachable mucus layer, the colon has two layers; one of which, the inner layer, is tightly attached to the epithelium. The single mucus layer of the small intestine is not impenetrable to bacteria, and invaders are kept away by means of antibacterial molecules. Crypts have the highest concentration of antibacterial compounds mostly produced by Paneth cells residing at the crypt bottom. Paneth cells are only found in the small intestine (As

reviewed in Mowat and Agace 2014). In the colon however, while the inner layer is impenetrable, the outer layer is penetrable and expanded, allowing bacteria to reside there (Johansson et al. 2008). In mouse caecum, the pouch-like structure considered the beginning of the colon where fermentation occurs, bacteria are in contact with the epithelium. This is not the case in the rest of the colon. The colon absorbs water and NaCl, which leaves the compact fecal material of micro-organisms and undigested fiber to mechanically stress the epithelium and simultaneously expose micro-organisms to it. Endogenous proteases cleave mucins in the inner layer, allowing their movement to the outer layer, lessening mucin density. This serves an important function; mucins can now cover fecal material and travel with it and micro-organisms in the outer layer. Because there is a lot of water in the mucin network, compounds do not diffuse away from it easily. Mucins are abundant in the small intestine and colon. In the small intestine, mucin-producing goblet cells are mainly found in crypts, but some can be found among enterocytes on the villi. In the colon, goblet cells are mostly found in upper parts of the crypt, and they are filled with large mucus granulae.

Major components in mucus are gel like glycoproteins called mucins that are highly o-glycosylated; more than 50% of mucin mass comes from o-glycans (As reviewd in Johansson and Hansson 2016). O-linked glycans give mucins their gel forming properties by being able to bind water. O-glycans can interact with cells and micro-organisms via their terminal glycans.

Mucins can be divided into two groups: transmembrane mucins and gel-forming mucins(As reviewed in Johansson and Hansson 2016). Transmembrane mucins have a single membrane spanning domain; the C-terminus is inside the cell and can have intracellular signaling properties, while the N-terminus is outside the cell on the apical side. There are numerous transmembrane mucins. MUC3, MUC4, MUC12, MUC13 and MUC17 are constitutively expressed. MUC3 is expressed throughout the intestine, but MUC12 is more specific to the colon. Transmembrane mucins cover the apical side of enterocytes.

Gel forming mucins that form the framework of mucus are secreted by goblet cells (As reviewed in Johansson and Hansson 2016). Goblet cells belong to the secretory lineage of intestinal stem cells and their maturation is partially controlled by transcription factor SAM pointed domain-containing Ets transcription factor (SPDEF) and Protein atonal homolog 1 (ATOH1), although other transcription factors are involved. MUC2 is the most abundant secreted mucin and it is expressed throughout the intestine; its production is highly stressful for goblet cells and at least two endoplasmic reticulum proteins are involved in its biosynthesis: Anterior gradient protein 2 homolog (AGR2) and endoplasmic reticulum to nucleus signaling 2 (ERN2). If constant hypersecretion of mucin occurs, unfolded proteins accumulate in the ER, which eventually leads to a decrease in secretion. The regulation of mucin biosynthesis is not completely understood, but it seems that translational control exists since discrepancy between mucin mRNA and protein levels are commonly seen.

MUC2 is packed in secretory vesicles as multimers; low Ca^{2+} and high pH keep them in ring like structures (As reviewed in Johansson and Hansson 2016). It is thought that there is a basal rate of release of mucus, but upon stimulation, goblet cells can empty their mucin storage and secrete it into the lumen, after which goblet cells are no longer identifiable since they don't contain any mucin. Because of this, it was wrongly thought that inflammation depleted goblet cells. A drop in Ca^{2+} and rise in pH causes the release of mucin by expanding them into net-like sheets. In the small intestine, this is achieved by cystic fibrosis transmembrane conductance regulator (CFTR), which takes up Cl^- and HCO_3^-. In the colon, it's not known how the secretion occurs. In the small intestine, outside the cell, meprin beta can digest the N-terminal of the mucin, which releases mucus from the epithelium. This makes the mucus layer porous enough for absorption of nutrients but at the same time it exposes the epithelium to the dangers of bacteria.

2.3.2 Impaired mucosal function in HSCR

Altered epithelial mucus has been shown be involved in the progress of HAEC (Thiagarajah et al. 2014). Abnormal mucin disrupts the epithelial barrier and leads to bacterial adherence. *Ednrb* null mutant mice exhibit short-segment HSCR and have increased numbers of goblet cells (hyperplasia) accompanied with a decrease in both neutral and acidic mucins in the distal colon. Exactly why this happens is not known, but it has been shown that enteric glia and neurons play a role in epithelial health (As reviewed in Sharkey and Savidge 2014).

In addition, *Ednrb*$^{-/-}$ mice have upregulated expression of transcription factors MATH1 and SPDEF in the distal colon but not in the proximal. MUC2 mRNA levels were unchanged but MUC4 mRNA levels were reduced in the distal colon. Changes in mucins have also been observed in humans (Aslam et al. 1997a; Aslam et al. 1997b; Aslam et al. 1999). Notably, an increase in ratio of the intracellular mucin to the secreted mucin is observed, indicating problems with mucin secretion (Aslam et al. 1998).

A recent study found that goblet cell related factors TFF3, SPDEF, and KLF4, but not MUC2, are downregulated in protein and mRNA levels in human patients in both aganglionic and ganglionic segments (Nakamura et al. 2018). Goblet cell related factors downregulated in the ganglionic segment might explain why dysmotility and HAEC occurs even after surgical removal of the aganglionic segment. In addition, the number of goblet cells was found to be reduced. These results conflict with findings from the HSCR model *Ednrb*$^{-/-}$ mice, except for similar MUC2 levels (Thiagarajah et al. 2014). It is possible that when HAEC progresses, goblet cells die due to secondary effects, which concurrently would lead to reduced mRNA detection of goblet cell related factors. In contrast, MUC2 protein levels observed in HSCR stool samples were found to be reduced (Mattar et al. 2003). A potential reason for this discrepancy might be that functionally secreted MUC2 is altered (Nakamura et al. 2018). In conclusion how mucin regulation affects HSCR and HAEC progression is currently not well understood.

2.3.3 Gut immune system

The intestine is constantly exposed to luminal antigens and substances included in diets that have modulating properties to the host microbiome (As reviewed in Mowat and Agace 2014). Epithelial cells express pattern recognition receptors (PRRs), such as toll-like receptors (TLR) and NOD-like receptors (NLR), both of which can be activated by luminal content and lead to immune responses. The intestine has specialized compartments called Gut-associated lymphoid tissue (GALT) that are responsible for immune system in the gut. Indeed, the intestine has more immune cells than any other tissue and most of the immunological processes occur in the mucosa, the inner most layer where many different cell types are involved in the complex process of immune response.

The intestine has a diverse population of T-cells (As reviewed in Belkaid et al. 2013). In normal conditions, the T-cell response is immunosuppressive, which is maintained through the production of TGF-β. TGF-β is produced mainly by antigen presenting cells (dendritic cells), and it favours development of the specific T-cell type T-regulatory (Treg) cells that suppress the gut immune system.

The majority of the B-cells in the intestine produce IgA-antibodies (As reviewed in Fagarasan and Honjo 2003). IgA which is secreted in to the lumen can trap viruses and bacteria and prevent their translocation though the epithelium, but it can also regulate microbial homeostasis.

Immune cells engage in an interplay with other cells like goblet cells in the epithelium; different interleukins can trigger hyperplasia or hypersecretion in goblet cells (As reviewed in Khan et al 2004; Oeser et al. 2015)

An appropriately working immune system is crucial for GI health (As reviewed in Johansson and Hansson 2016). The cells of the immune system produce a variety of different cytokines like tumor necrosis factor alpha (TNFα), interferon gamma (INF**γ)**, interleukin 1 beta (IL1β), interleukin 6 (IL6), interleukin 10 (IL10). Immunodeficient mice are more disposed to develop colitis spontaneously or have increased likelihood of developing it after dextran sulphate sodium (DDS) administration. Of all cytokines,

IL10 is the most extensively studied interleukin in relation to colitis and the pathogenesis of the IL10-related colitis seems to involve the mucin layer.

2.3.4 Immune dysfunction in HSCR

Changes in immune cells and different antibodies produced by B-cells have been observed in HSCR patients with enterocolitis (Imamura et al. 1992). Levels of IgA, IgM but not IgG, producing plasma cells were increased in the lamina propria. However, the luminal IgA content was reduced, which might predispose one to enterocolitis.

EdnrB$^{-/-}$ animals with an aganglionic segment of the distal colon have reduced spleen size, which was characterized by a reduction in CD19^{+} mature B-cells, CD4^{+} T-cells, and CD8^{+} T-cells (Frykman et al. 2015a). The authors concluded that the effect seen is not caused by a lack of endothelin signaling, but rather by a lack of ENS innervation that causes intestinal obstruction since a bone marrow transplant from *EdnrB*$^{-/-}$ mice to RAG2-null mice was able to repopulate recipient bone marrow and the mice would not develop enterocolitis later. RAG2-null mice are immunodeficient because Recombination activating gene 2 (RAG2) is an essential gene for lymphocyte maturation as it is involved in process of V(D)J recombination.

Abnormal immunity is also seen in the small intestine away from the aganglionic segment (Pierre et al. 2014). *EdnrB*$^{-/-}$ mice at P22 have reduced expression of antimicrobial proteins secreted by Paneth cells, possibly compromising innate immunity in the small intestine. Dysfunction in intestinal immunity which occurs far away from the aganglionic segment might explain why the threat of HAEC still exists after successful surgery.

It is well documented that the ENS communicates with the intestinal immune system (Ibiza et al. 2016; as reviewed in Margolis et al. 2016). Enteric fibres innervate mucosa which enable interaction with immune cells that express receptors for neurotransmitters. An abnormal ENS might be involved in the development of HAEC and it's been shown that HSCR patients have immature neurons in the normoganglionic segment (Miyahara

et al. 2009). Another study found deficient mucosal innervation in transitional segments that contained ganglia (Wendelschafer-Crabb et al. 2009).

2.3.5 Gut microbiome

The GI microbiome has recently been intensively studied (As reviewed in Cani 2018). In fact, more than 80% of all papers published on the subject since 1977 were published between the years 2013 and 2017. Now, the gut microbiome has been linked to several different diseases from cardiometabolic disorders to psychiatric disorders. It was estimated that the human gut has 10^{14} microbes, which is ten times the number of all human cells (As reviewed in Backhed et al. 2005; Gill et al. 2006). A more recent study, however, estimated ratio to be 5:1 (Sender et al. 2016). Nevertheless, gut bacteria are numerous and appear to be important regulators of physiological processes in the body.

In the colon, the loose outer layer of mucus allows bacteria with their adhesins to interact with mucin glycans; different mucin glycans among different species seem to affect what kind of bacteria resides there (Johansson et al. 2008; as reviewed in Johansson and Hansson 2016). It is exactly the outer layer of mucus where commensal bacteria reside. Some of commensal bacteria can use glycans as their sole source of energy, but most bacteria prefer undigested polysaccharides, meaning the host's diet impacts the bacterial composition. Commensal bacteria produce important metabolites such as short-chain fatty acids (SCFAs). The SCFA butyrate is especially important, and a decrease in butyrate has been implicated in colon cancer and inflammation. It should be noted that, butyrate is very important for goblet cell to produce MUC2, as it becomes their primary source of energy when other energy sources are limited.

2.3.6 Abnormal microbiome in HSCR

An abnormal microbiome might contribute to HAEC (Frykman et al. 2015b; as reviewed in Gosain 2016). Supporting this, antibiotic treatment with metronidazole reduces HAEC symptoms. Also, it has been shown that differences in SCFA-producing bacteria have been observed in HAEC patients (Demehri et al. 2016). Still, conclusive data about the microbial origin for HAEC does not exist. Lately, it was shown that *Ednrb*$^{-/-}$ mice that develop spontaneous enterocolitis had increased numbers of bacteria that might play a role in HAEC (Cheng et al. 2018). Figure 3 summarizes different abnormalities that are thought to be involved in HAEC. Currently, the sequence of events leading to HAEC is unclear, as also can be derived from the lack of spatial and temporal etiological information in the below scheme (Figure 3).

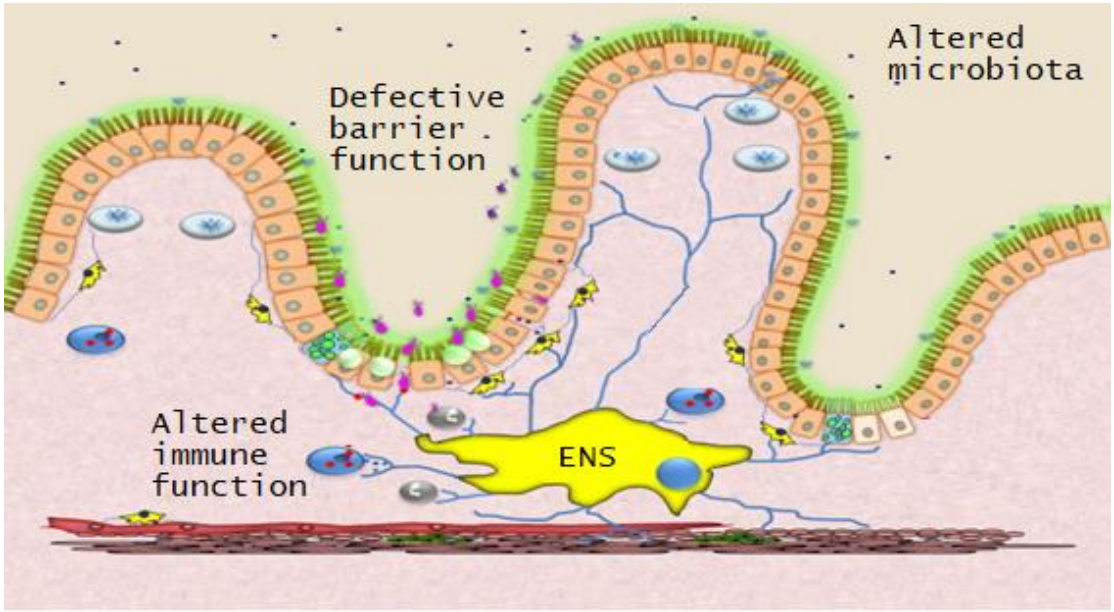


Figure 3. The interactions in the gut wall. The ENS regulates all aspects of intestinal homeostasis, and aberrant control is thought to predispose HAEC. Different functions that are disrupted in HSCR are shown in the figure (As reviewed in Austin, 2012).

3 Glial cell line–derived neurotrophic factor

Neurotrophic factors (NTFs) including neurotrophins, glial cell line–derived neurotrophic factor (GDNF) family ligands (GFLs), and neuropoietic cytokines are a class of small secretory proteins or peptides that have specific receptors on the cell surface that activate intracellular signalling pathways (As reviewed in Boyd and Gordon 2003). NTFs regulate virtually all aspects of the life of a neuron: they promote the survival, migration, and differentiation of neurons, making them particularly important during development but also in adulthood. NTFs can stimulate neurite outgrowth and axonal (re)generation and can increase synaptic plasticity. Also, their neuroprotective properties have been studied extensively in neurodegenerative diseases such as Parkinson's disease. In animal models of Parkinson's disease, GDNF, famous for promoting survival and differentiation of dopaminergic neurons, has shown promising results. As a RET ligand, GDNF, in conjunction with its co-receptor GDNF family receptor alpha-1 (GFRα1), is also an extremely important regulator of ENS development, regulating the survival, proliferation, and migration of enteric neuron precursor cells (Gianino et al. 2003; Mwizerwa et al. 2011). Consequently, knockouts of *Gdnf*, *Gfra1* or *Ret* lack ENS innervation (Enomoto et al. 1998; Sánchez et al. 1996; Schuchardt et al. 1994). To date, researchers have failed to generate postnatally viable mouse models of HSCR that affect GDNF/GFRα1/RET signalling likely because GDNF/GFRα1/RET signalling is also essential for kidney development and pups with gene deletion or severe mutations in the encoding genes are born without kidneys and die soon after birth. Less impairing mutations in genes encoding for GDNF/GFRα1/RET on the other hand result in normal or close to normal ENS without HSCR and HAEC.

GFLs belongs to the transforming growth factor-β (TGF-β) superfamily (As reviewed in Airaksinen and Saarma 2002). The structure of GDNF contains seven cysteine residues and it has two sites of glycosylation. GDNF is a homodimer; it has a structural motif called a cystine knot that aids the formation of the dimer via cysteine bridges that stabilize the protein conformation. Indeed, all the proteins in the TGF-β superfamily have seven cysteine residues that are spaced in a relatively similar way. Interestingly, proteins in this family do not have high amino acid homology, yet they have a similar

conformation. GDNF is translated as preproprotein. The signal sequence (pre) is cleaved during secretion and finally proGDNF is enzymatically digested to yield mature GDNF protein.

GDNF like all other GFLs mediate their effects primarily through the RET receptor tyrosine kinase, unlike other TGF-β superfamily proteins that mediate their effects through serine-threonine kinases(As reviewed in Airaksinen and Saarma 2002). However, GFLs do not bind to RET alone; rather, they bind to their preferred co-receptor GFRα1-4 first, and then the complex is able to bind to RET. GDNF binds preferentially to the Gfrα1 co-receptor (Figure 4). However, there is evidence that some cross talk exists between other GFL co-receptors; for instance, GDNF is able to also bind to Gfrα2 and Gfrα3. Gfrαs can be anchored to membrane via glycosylphosphatidylinositol (GPI) links and they consist of 3 globular cysteine rich domains (except for, Gfrα4, which has only 2 domains). Domains 2 and 3 are involved in RET binding. RET is a single spanning transmembrane protein that has four extracellular cadherin-like domains of which one has a binding site for Ca^{2+}, that is essential for RET activation. GDNF/GFRα1 complex interacts with RET causing the homodimerization and subsequent autophosphorylation of specific tyrosine residues in the RET kinase domain, leading to signal transduction in the cell.

Phosphorylated tyrosines in RET can serve as docking sites for intracellular signalling proteins Src homology domain (Shc) and Fibroblast growth factor receptor substrate 2 (FRS2), which then activate several signalling pathways such as the RAS/Mitogen activated protein kinase (RAS/MAPK), phosphatidylinositol 3-kinase/Akt (PI3K/Akt), and Phosphoinositide phospholipase C (PLCγ) pathway (As reviewed in Airaksinen and Saarma 2002). Depending on the cellular context, these pathways play an important role in proliferation, adhesion, migration, differentiation and survival.

GFRα1/RET signal transduction can occur by means of cis-signalling or trans-signalling (As reviewed in Airaksinen and Saarma 2002). In cis-signalling, GFRα1 is anchored to the lipid raft and the signalling occurs in the same cell where GFRα1 is anchored as seen in figures 9 and 10. Alternatively GDNF can bind to freely floating GFRα1 . This is possible because GFRα1 is constitutively cleaved off the membrane by intracellular phospholipases, which release GFRα1 and enable non-cell-autonomous signalling.

Freely floating GDNF/ GFRα1 complexes can activate signalling pathways on RET-expressing cells.

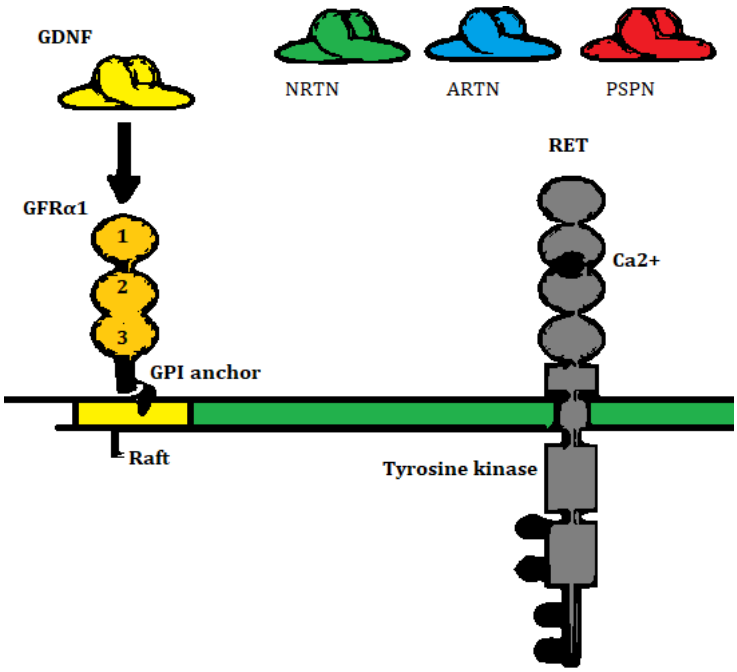


Figure 4. In order to signal, GDNF must first bind to its preferred co receptor GFRα1 as shown in the figure. GFRα1 have three cysteine rich domains. The GFL-GFRα complex is formed usually when GFL binds domain 2, which is very important for RET binding as well. In addition, Ca^{2+} is required for RET activation. The intracellular domain of RET consists of four tyrosine residues that facilitate the docking of different adaptor proteins. GFRα1-receptor is anchored to lipid rafts on the membrane by glycosyl phosphatidylinositol (GPI). The other GFLs Neurturin (NRTN), Artemin (ARTN) and Persephin (PRSN) bind to their cognate co receptors GFRα2, GFRα3 and GFRα4 respectively (not shown in figure). (Modified from Airaksinen and Saarma 2002).

3.1 Enteric nervous system development and GDNF signalling

Development of the ENS is very complex; starting population of 1000-2000 cells give rise to over a million enteric neurons and even more glial cells in adult mice small

intestine and colon (As reviewed in Anderson et al. 2006a). For this to happen, developing enteric neurons must migrate and proliferate, but some enteric neurons also have to differentiate at the right time while others are still proliferating and migrating. Moreover, many signaling pathways are required for the development of a properly functioning ENS especially, GDNF/GFRα1/RET and endothelin-3 (ET3) – EDNRB. The GDNF/GFRα1/RET signalling pathway has exceptionally great importance in ENS development as it controls major aspects of enteric neuron development: survival, proliferation, migration, and differentiation.

The formation of the ENS is initiated when cells called neural crest cells (NCCs) delaminate from neural tube at E8.5 and enter the foregut one day later (As reviewed in Anderson et al. 2006a; Durbec et al. 1996). NCCs originate from the ectoderm layer formed during gastrulation, and they can give rise to several different cell lineages, such as enteric neurons and glia, but also melanocytes, craniofacial cartilage, bone, and smooth muscles (As reviewed in Bronner et al. 2012). The majority of NCCs that give rise to the ENS originate from levels 1-7 of somites (Figure 5) (Le Douarin and Teillet 1973)). These NCCs are called vagal NCC-derived cells and it was shown that in the chick embryo, enteric ganglia were missing after ablation of vagal NCC-derived cells (Yntema and Hammond 1954).

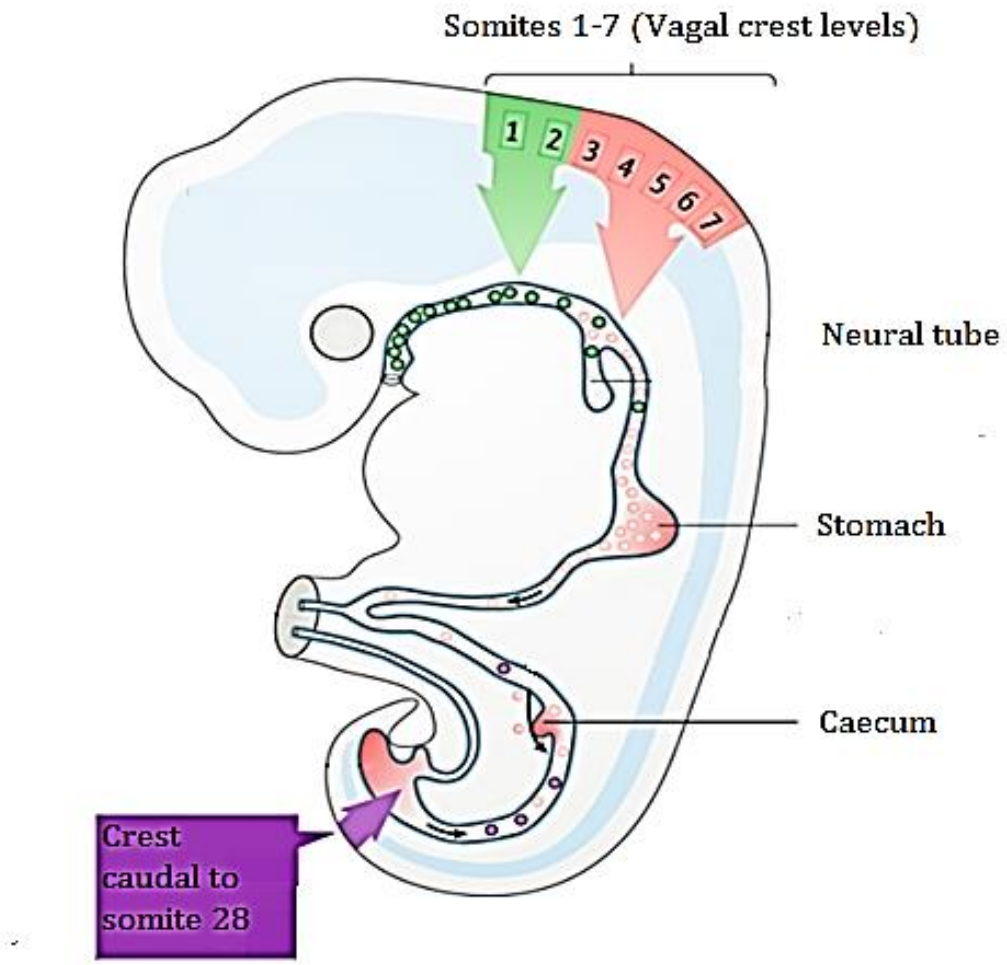


Figure 5. Formation of the ENS. Primordial gut is colonized by vagal neural crest derived cells that correspond to somite levels 1-7. These cells enter the foregut at E9.5 and migrate rostro-caudally along the gut (arrow shows the direction of the migration). Sacral neural crest derived cells that originate from somite level 28 colonize a small segment of the distal gut. Their entry into the hindgut coincides with vagal neural crest derived cells arrival. (Modified from Rao and Gershon 2018)

Before entering the foregut, vagal NCC-derived cells, like all other neural crest derived cells, express the transcription factor SOX10 (Kuhlbrodt et al. 1998). Initial expression of Sox10 is necessary for the progenitors of the developing ENS as it promotes their survival and maintains their potency (Britsch et al. 2001; Herbarth et al. 1998; Lane and

Liu, 1984; Southard-Smith et al. 1998). This is highlighted by the fact that *Sox10*^{Dom} homozygous mice have total aganglionosis of the gut because of early apoptosis of neural crest cells. *Sox10*^{Dom} is a naturally occurring autosomal dominant mutation with incomplete penetrance. The necessity of Sox10 expression in early survival of enteric progenitors is most likely due to its necessity for RET expression. Apoptosis in the subpopulation of enteric progenitors is observed in RET null mice shortly after entering the foregut (Taraviras et al. 1999). PHOX2B is also required for RET expression at later stages, but earlier RET expression is independent of PHOX2B as its expression starts when vagal NCC-derived cells enter the gut and start their migration (Pattyn et al. 1999). GDNF-Ret dependent survival of vagal NCCs seem to be mediated by PI3K/Akt/Forkhead pathway (Srinivasan et al. 2005). FOXO1 mediated cell death is interrupted when PI3K phosphorylates Akt which then in turn phosphorylates FOXO1 and FOXO3a leading to translocation of these transcription factors out of the nucleus. It should be noted that Neurturin, another RET ligand, also has a role in ENS development (Gianino et al. 2003). However, the co-receptor for Neurturin GFRα2 is not expressed until E14, so Neurturin doesn't play a role in early development, but it is an important trophic factor regarding the survival of mature myenteric neurons and inactivating mutation in this gene cause hypoganglionosis of myenteric plexus. Few cases of HSCR have been reported where mutation is has been found in *GDNF* or *NRTN*.

As vagal NCC-derived cells enter the foregut they are called enteric neural crest derived cells (ENCCs) and they start expressing markers committing them to enteric glial and neuronal lineages (As reviewed in Nagy and Goldstein 2017). In mice, for ENCCs to colonize the whole gut, which is achieved in 4 days (E9.5-E13.5), the migrating precursor pool must maintain a sufficient number of progenitors, but at the same time, timely differentiation to enteric neurons and glia must occur. Thus, balance between proliferation and differentiation must be tightly regulated.

Besides being a critical factor for early survival and migration of enteric progenitors, GDNF/GFRα1/RET signalling stimulates proliferation of ENCCs ensuring that cell number is sufficient for colonization of the whole gut (Focke et al. 2001). PI3K/Akt/ mediates also GDNF stimulated proliferation of ENCCs. This effect is blocked by a selective PI3K inhibitor in primary cultures of ENCCs. In the same experiment, inhibition of the extracellular-signal-regulated kinase (ERK), a component of the

MAPK pathway had no effect on proliferation. Indeed, in the early stages of ENS development, GDNF acts as a proliferating signal as ENCCs are still migrating which happens synergistically with ET3 (Barlow et al. 2003). However, later in the development, GDNF stimulates migration and promotes neuronal differentiation whereas ET3 inhibits both migration and neuronal differentiation (Hearn et al. 1998; Mwizerwa et al. 2011; Wu et al. 1999; Nagy and Goldstein 2006).

The time when a neuronal precursor exits the cell cycle is defined as the birth of that neuron (As reviewed in Hao and Young 2009). It's thought that in the CNS, neuronal precursors exit the cell cycle before starting to express pan-neuronal markers, but in the ENS, like sympathetic neurons, neural crest derived cells have the capability to divide after expressing pan-neuronal markers such as Neuron-specific class III beta-tubulin (TuJ1).

ENCCs can differentiate either into enteric neurons or glial cells (Figure 6) (Paratore et al. 2001; Kim et al. 2003). SOX10, an essential transcription factor in the early stages of ENS development, determines yet another important aspect of ENS progenitors. In order to generate glial cells, Sox10 expression needs to be maintained. However, enteric neuron differentiation requires Sox10 downregulation. Concurrently, RET expression needs to be maintained in the cell lineage that gives rise to enteric neurons but, in the glial cell lineage, RET must be downregulated. The pathway that underlies GDNF-RET mediated differentiation of neurons seems to be the MAPK/ERK pathway, and low levels of ERK activation indicated by minimal pERK prevail in undifferentiated neuronal precursors (Uesaka et al. 2013).

The migration of ENCCs occurs rostro-caudally at the speed of about 40 μm/h in a chain-like manner where cells communicating with each other enhance the migratory behavior (Figure 7) (Young et al. 2004). The L1 cell adhesion molecule (L1CAM) is an important cell adhesion molecule for ENS migration that keeps cells in contact with each other and 1% of HSCR patients indeed have mutations in *L1CAM* (Anderson et al. 2006b; As reviewed in Heuckeroth et al. 2018). Experimental evidence indicates that isolated ENCCs do not migrate as fast and their directionality is also altered, emphasizing the importance of chain migration. It has also been shown that if the starting population of ENCCs is experimentally reduced, aganglionosis of the distal gut

occurs (Peters-van der Sanden et al. 1993). As a lower ENCC density results in a slower migration speed, further colonization may be hindered because the target microenvironment has already become nonpermissive to further migration (Druckenbrod et al. 2009).

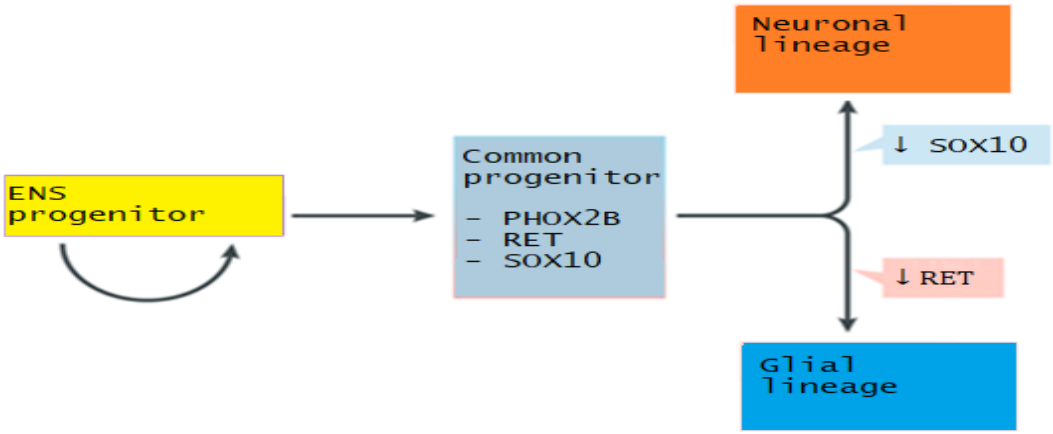


Figure 6. From ENS progenitor to enteric neurons/glia. ENS progenitors will give rise to all the enteric neurons and glial cells in the gut and the decision to become a neuron or glial cell is depended on SOX10 and RET. The lineage that gives rise to enteric neurons will have to downregulate SOX10 and maintain RET expression. In contrast, enteric glial cells retain SOX10 but downregulate RET (Modified from Rao and Gershon 2018).

Migrating cells are not a homogenous population; cells closer to the wavefront express Sox10, RET, PHOX2B, but cells trailing behind are more heterogenous in their expression pattern as they are in a different stage of maturation (Young et al. 2004; as reviewed in Lake and Heuckreroth 2013). These immature neurons project neurites that might affect the migratory behavior of the cells at the wavefront. When migrating cells

behind the head of the wavefront are isolated their migratory speed is slower than the migratory speed of original population (Young et al. 2004). This indicates that high cell density and cell/cell contacts are a driving force for migration and colonization ("population pressure").

When the wavefront is approaching the cecum, circular smooth muscle layer starts to form, restricting ENCC migration between the circular muscle layer and serosa (As reviewed in Nagy and Goldstein 2017). This is exactly where myenteric ganglia responsible for gut motility will form. Later, a second wave of migration called radial migration occurs. Radial migration occurs when the myenteric plexus is formed, and the subset of cells migrate radially from the myenteric plexus towards the epithelium forming the submucosal plexus.

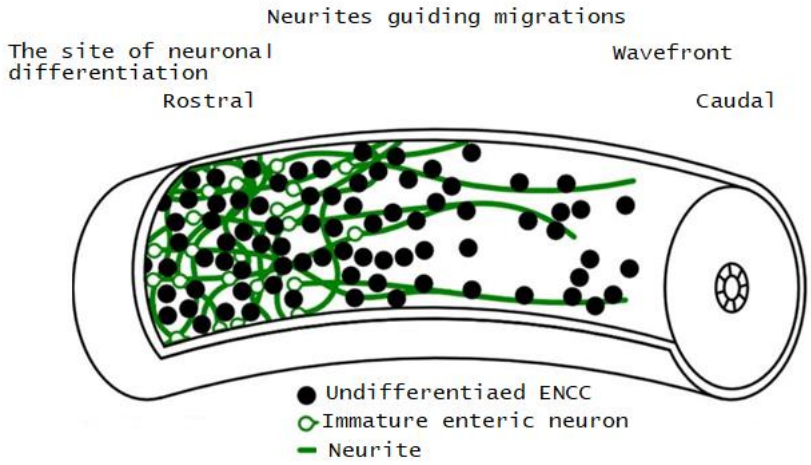


Figure 7. Migratory pattern in ENS development. Migrating cells are connected to each other via adhesion molecules such as The L1 cell adhesion molecule (L1CAM), enhancing their migratory behavior. Cells in the wavefront are undifferentiated ENCCs while cells behind the waverfront have become immature neurons that project neurites along wavefront. (Modified from Lake and Heuckreroth 2013).

GDNF has a major impact on the migration of enteric neural crest cells (ENCCs), and it is an essential driver of rostro-caudal migration of ENCCs (Natarajan et al. 2002).

GDNF acts as a chemoattractant for ENCCs and high levels of *Gdnf* mRNA are expressed in the mesenchyme of the foregut before the arrival of *Ret* expressing ENS precursor cells. Later, GDNF expression is seen in the cecum when migrating ENCCs are still in the midgut. Again, the PI3K/Akt pathway plays a key role as this pathway activates the expression of *Rac1* at the wavefront (Guo et al. 2006). RAC1 is key component in lamellipodia formation and cell motility .One of the counter regulators of this pathway is the protein phosphatase and tensin homolog (PTEN). PTEN converts Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) back to Phosphatidylinositol 4,5-bisphosphate (PIP_2), thereby reducing the effects of PI3K.

Besides being critical regulator of rostro-caudal migration, GDNF signaling is also required for radial migration (Uesaka et al. 2013). This was demonstrated by Uesaka et al. who showed an almost total absence of submucosal ganglia after conditionally knocking out *Gfra1* at E15.5 from $Gfra1^{GFP/+}$ mice that express the green fluorescent protein (GFP) from the Gfra1 locus. Analysis 3 days afterwards revealed that neurons that would normally form the submucosal plexus were mostly missing.

Finally, a smaller portion of the ENS comes from sacral NCC-derived cells that are caudal to somite 28 (Burns and Douarin 1998; as reviewed in Nagy and Goldstein 2017). In mice, Sacral NCCs colonize the hindgut, the most distal part of the gut. It has been estimated that these cells make up to 17% of the total enteric neurons in the terminal hindgut, but only 0.3% in the proximal hindgut. It is not known how much the sacral NCCs contribute to the ENS in humans. Sacral NCCs enter the hindgut later when vagal NCCs have already arrived there. Sacral NCCs are less invasive than vagal NCCs and this can be seen when the sacral neural tube is replaced by the vagal neural tube. In this setting, vagal NCCs will colonize even more proximal parts of the hindgut. An explanation for the difference in their migration behaviour might lie in their difference in RET expression (Pachnis et al. 1993). Microarray analysis revealed that out of 11 important ENS regulator genes, only RET expression was altered; the expression of RET was 4-fold higher in Vagal NCCs. Thus, it is possible that sacral NCCs might not respond to GDNF at all.

4 Conclusion

The ENS is the most complex neural network of the peripheral nervous system (PNS), and it can independently control gut function as Bayliss and Starling showed already in 1899 (Bayliss and Starling 1899). ENS dysfunction is thought to underlie many gut related pathologies, and it may be that at least in some cases dysfunction arises already during its development, which is a highly elaborate process that requires spatiotemporal coordination between signalling pathways which control migration, proliferation, and differentiation (As reviewed in Lake and Heuckeroth 2013). This is evident in the complex pathophysiology of HSCR, as dysregulation of various genes involved in ENS development result in disease. Yet, despite decades of active research, many of the HSCR susceptibility genes and mechanisms that underlie it are still not known. Moreover, environmental factors can influence or even disrupt the process of ENS development. This includes common drugs such as non-steroid anti-inflammatory drugs or selective serotonin inhibitors (SSRI) (As reviewed in Nijenhuis et al. 2012; Schill et al. 2016).

Aganglionosis can be successfully treated by the resection of the aganglionic GI segment, but the risk of post-operatively acquiring the most fatal complication, HAEC, is still high (As reviewed in Frykman et al. 2012). This implies that in the ganglionic segment of HSCR patients the ENS does not function normally. Therefore, the elaborate interaction between the ENS and the gut requires further research.

Currently, the exact mechanisms behind HAEC remain mostly unknown, but studies have shown that abnormal mucin production, immune dysfunction, and altered microbiome might be involved (Pierre et al. 2014; Thiagarajah et al. 2014; Cheng et al. 2018). However, whether these factors are causes or consequences is still not known. Pinpointing the exact order of the sequence of events would greatly help to understand the progress of HAEC.

GDNF-GFRα1-RET signalling is of special interest in studying HSCR as RET mutations are overwhelmingly the most common cause of HSCR accounting for circa 50% of all familial cases (Attié et al. 1995; as reviewed in Kenny et al. 2010). The reason for this might not be surprising given the multifaceted effects of RET during

ENS development. However, kidney agenesis resulting from knocking-out of *Gdnf*, *Gfra1* or *Ret* mice excludes the use of these mice as models of postnatal HSCR (Enomoto et al. 1998; Sánchez et al. 1996; Schuchardt et al. 1994).

In summary, studying the role of GDNF/GFRα1/RET signalling in the development and pathophysiology GI aganglionosis is crucial to improve our understanding of HSCR and provide, eventually, targeted treatments to HAEC patients.

II: EXPERIMENTAL PART

1 INTRODUCTION

The aim of the experimental part of this thesis is to shed light on the order of the features of early HAEC by using a GFRa1 hypomorphic mouse model (Porokuokka et al. 2018). Reduced *GFRA1* have been reported in human infants suffering from HSCR (Lui et al. 2002). However, whether reduced GFRa1 expression can result in HSCR and/or HAEC has remained unknown. GFRa1 hypomorphic mice were created by inserting an antibiotic resistance cassette within intron six of the *Gfra1* gene. As a result, there is a 70-80% reduction in *Gfra1* gene expression in the developing gut and kidney. Despite this reduction, the kidneys still develop normally, allowing postnatal analysis. Nevertheless, GFRa1 hypomorphic mice are born with long-segment HSCR. This is the first successful model that manipulates GDNF-GFRα1-RET signalling and creates a postnatally viable mouse model of HSCR and HAEC. These HSCR/HAEC mice are usually sacrificed between P7-P25 due to welfare issues.

Current knowledge reported about HAEC involves various changes in the gut epithelium, especially in mucin-producing goblet cells (Thiagarajah et al. 2014; Nakamura et al. 2018; Porokuokka et al. 2018). These changes include hyperplasia of the goblet cells, altered mucin profile, retention of mucin, damaged and disorganized epithelium structure, inflammation, and bacterial adherence into the epithelium. However, shortage of suitable postnatal HSCR mouse models have partially hindered

the progress of pinpointing the exact order these events. The main focus of this thesis is to analyse the molecular and histological changes in HSCR/HAEC progression in GFRa1 hypomorphic mice. In our long segment HSCR GFRa1 hypomorphic mice, the distal colon is the segment that is usually aganglionic. Still, in some animals the proximal parts of the colon are also affected, and in few individuals, the affected segment extends to distal ileum. In addition, I also studied several inflammation markers locally in the gut and systemically in the blood.

Goblet cells are part of the body's first line of defence against invading pathogens, linking goblet cells and the immune system in a tight interplay (Johansson et al. 2008). For example, the regulatory region of *MUC2*, which is the most abundantly expressed mucin, has a binding site for nuclear factor kappa-light-chain-enhancer of activated B cells protein complex (Nf-κB) (Ahn et al. 2005). Nf-κB is a protein complex involved in inflammation that can be activated by cytokines such as TNFα. Interestingly, depending on contextual conditions, TNFα can both upregulate and downregulate *MUC2* transcription. TNFα mediated upregulation of *MUC2* transcription occurs through Nf-κB activation, but TNFα can also activate the c-Jun N-terminal kinase (JNK) pathway, having a negative effect on *MUC2* transcription.

Goblet cells produce different types of mucins that can be visualized histologically by using AB-PAS staining (Cohen et al. 2012). AB-PAS is a staining technique that combines two different stains: Alcian blue (AB) and Periodic Acid-Schiff base (PAS). AB is a basic dye that stains the acidic mucin and PAS stains the neutral mucins. Using these in conjunction makes visualization of the composition of mucins possible at a given time point. Changes in neutral and acidic mucins have been observed not only in inflammatory GI diseases such as Crohn's disease and ulcerative colitis, but also in Hirschsprung's disease (Rhodes et al. 1985; Thiagarajah et al. 2014; Nakamura et al. 2018). However, the exact mechanism behind the change in mucin profile is not well understood.

The gut has the most immune cells in the whole body and it is constantly exposed to different luminal antigens (As reviewed in Mowat and Agace 2014). This sets a high requirement for proper control of the immune system; immune cells need to be active enough to fend against bacterial invasion, but not too active to inappropriately cause

excess inflammation in the absence of a threat. The immune system is immensely complex, involving numerous different cytokines secreted by various cell types in communication with each other. Disruption of this communication can lead to excessive inflammation, and in the gut this can compromise the epithelial integrity, which might have critical implications on GI tract function.

2 MATERIALS AND METHODS

2.1 Animals

Mice with mixed C57BL6/129ola background were used. GFRa1 hypomorphic mice were created by Dr. Jaan-Olle Andressoo. A positive/negative selection cassette puΔtk was inserted within intron six of the *Gfra1* gene via homologous recombination in embryonic stem cells (ES) (Figure 8.). Since homozygous *Gfra1*$^{hypo/hypo}$ mice die before becoming fertile, two heterozygous *Gfra1*$^{WT/hypo}$ mice were bred to generate homozygous *Gfra1*$^{hypo/hypo}$ mice. The mice were housed in a 12h/12h light/dark cycle at 20-22°C and had ad libitum access to standard food and water.

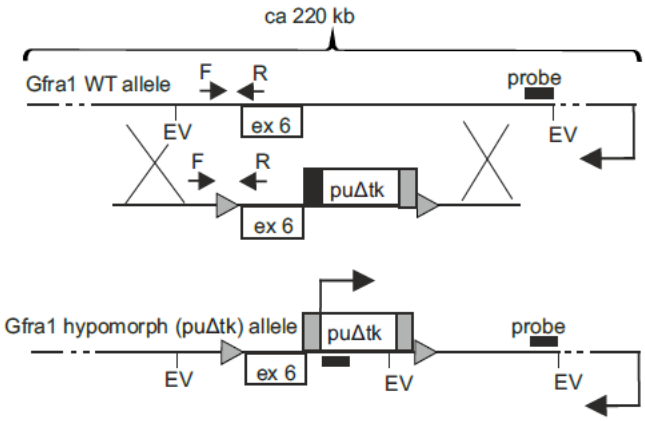


Figure 8. *Gfra1* hypomorph allele. Transcription of the puΔtk cassette within intron 6 of the *Gfra1* gene interferes with *Gfra1* transcription. As a result, the hypomorphic allele is created. LoxP sites are indicated as triangles.

2.2 Tissue processing

Whole mount tissue samples were collected from the colon and duodenum and placed in freshly made 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight. On the next day, the tissues were dehydrated and embedded using an ASP300 S tissue processor (Leica). 5-µm paraffin sections were cut using the Tissue-Tek microtome (Sakura).

2.3 Histology

A combination of Alcian blue (pH 2.5) and periodic acid–Schiff base was used to visualize and subsequently quantify mucins in the colon and duodenum. Standard AB-PAS staining protocol was used.

2.3.1 Histological quantification

To quantify the ratio of acidic mucin:neutral mucin, the goblet cells in the distal third of the colon were counted. Only goblet cells in the lower half of the crypt were counted and, based on their colour, they were counted as alcian blue positive (AB+) if mostly light blue or deep blue, or periodic acid–Schiff positive (PAS+) if mostly magenta or purple. If the cell couldn't be put in either of the two categories, it was as AB/PAS+.

The same quantifying system was used in the proximal duodenum, but instead of counting the cells in the crypt where there are few goblet cells, goblet cells in the villi were counted. In addition, the total number of the goblet cells was counted. In the colon, goblet cells were counted in the whole length along the crypts in the distal third of the colon. In the proximal duodenum, goblet cells were counted in villi.

2.4 ELISA

The blood was collected by cardic puncture or the mouse was decapitated, and the blood was spilled on parafilm from which it was collected with pipette. The blood was then spun at 8000 rpm for 3min and the serum was collected; this procedure was repeated once.

To perfom the analysis of serum cytokines, three Invitrogen kits were used: Invitrogen ProcartaPlex™ multiplex IFNg, IL12, IL4, IL5, IL6, TNFa, EPX060-20831-90, IL-1b Mouse ProcartaPlex™ Simplex Kit, EPX01A-26002-901, and IL-9 Mouse ProcartaPlex™ Simplex Kit, EPX01A-26041-901

2.5 RNA isolation

Samples from the distal colon and small intestine of E18.5-P14 mice for qPCR-analysis were snap-frozen immediately, and RNA from the snap-frozen tissues was isolated using RNAqueous Micro-kit (AM1931, Life Technologies) in accordance with the manufacturer's instructions.

2.6 Complementary DNA (cDNA) synthesis

400-500ng of RNA in 8 µl was used to synthesize cDNA. First, the samples were treated with DNase to eliminate genomic DNA impurities. Then, 1 µl of EDTA was added to prevent DNase digestion of cDNA. In the final step, 4 µl of reverse transcriptase (RT) buffer, 2 µl of dNTPs, 1.1 µl of dH20, 1 µl of random hexamer primers, 0.5 µl RT, and 0.4 µl of RNase inhibitor were added to create cDNA. For the negative control, all the above was added except RT. The final volume of 20 µl was diluted 1:10, and then either used immediately for qPCR or stored −20°C.

2.7 Quantitive real-time PCR (qPCR)

LightCycler 480 real-time PCR system (Roche Diagnostics) was used to perform the qPCR. For each reaction 2.5 µl of cDNA, 7.5 µl of LightCycler 480 SYBR Green I Master mix, and 2.5 pmol of primers were used. The samples were pipetted in 2-3 replicates into 384-well plates sealed with an adhesive plate sealer (04729749001, Roche Diagnostics). The qPCR program had the following settings: pre-incubation for 10 min at 95°C; amplification for 10 s at 95°C, 15 s at 60°C, and 15 s at 72°C for 45 cycles; melting curve for 5 s at 95°C and 30 s at 55°C; continuous acquisition mode at 95°C with two acquisitions per degree Celsius; and cooling for 10 s at 40°C. For quantification, beta-actin was used as a reference gene. LightCycler 480 Software Release 1.5.0 SP1, with the Absolute Quantification/2nd Derivative Max calculation was used to analyze the data. The primers that were used in the study are as follows:

mActb 5'-ctaaggccaaccgtgaaaag 5'-accagaggcatacagggaca

mAtoh1 5'-tgcgatctccgagtgagag 5'-tctcttctgcaaggtctgatttt

mGfra1 5'-ttcccacacacgttttacca 5'-gcccgatacattggatttca

mGlcnac6st2 5'-ggggagcagctatcacga 5'-ccctttcttcaacagcatcat

mIfng 5'-ttcttcagcaacagcaaggc 5'-tcagcagcgactccttttcc

mIlb 5'-agttgacggaccccaaaag 5'-agctggatgctctcatcagg

mIl1a 5'-gtcggcaaagaaatcaagatg 5'-gtcttcgttttcactgtaacag

mIl2 5'-ttgtgctccttgtcaacagc 5'-ctggggagtttcaggttcct

mIl4 5'-aacgaggtcacaggagaagg 5'-tctgcagctccatgagaaca

mIl5 5'-accgagctctgttgacaag 5'-tcctcgccacacttctcttt

mIl6 5'-accacttcacaagtcggagg 5'-tgcaagtgcatcatcgttgt

mIl10 5'-ataactgcacccacttccca 5'-cttggcaacccaagtaaccc

mIl13 5'-gcagcatggtatggagtgtg 5'-tggcgaaacagttgctttgt

mIl23 5'-gctgtgcctaggagtagcag 5'-tggctgttgtccttgagtcc

mMuc2 5'-caagtgattgtgtttcaggc 5'-tggagatgttcttggtgcag

mMuc4 5'-gagggcctcactggagagtt 5'-tctgtgttgatgttgttgctagag

mPgp9.5 5'-tgctcctgtttcccctca 5'-cctgtcccttcagttcctca

mSpdef 5'-gatgtactgcatgcccacct 5'-ggaggcgcagtagtgaagg

mTgfb1 5'-tggagcaacatgtggaactc 5'-gtcagcagccggttacca

mTnfa 5'-gatcggtccccaaagggatg 5'-tgagggtctgggccatagaa

3 RESULTS

3.1 Characterization of the *Gfra1*$^{hypo/hypo}$ GI tract

Due to puΔtk cassette insertion, homozygous *Gfra1*$^{hypo/hypo}$ mice have 75-80% reduction in Gfra1 mRNA expression in the developing gut and kidney. Apparently, this Gfra1 mRNA dose allows kidneys to develop normally but results in aganglionosis in the colon. Massive GI distention because of intestinal obstruction is observed due to aganglionosis (Figure 9). *Gfra1*$^{hypo/hypo}$ mice have congenital features that resembles childhood long-segment HSCR.

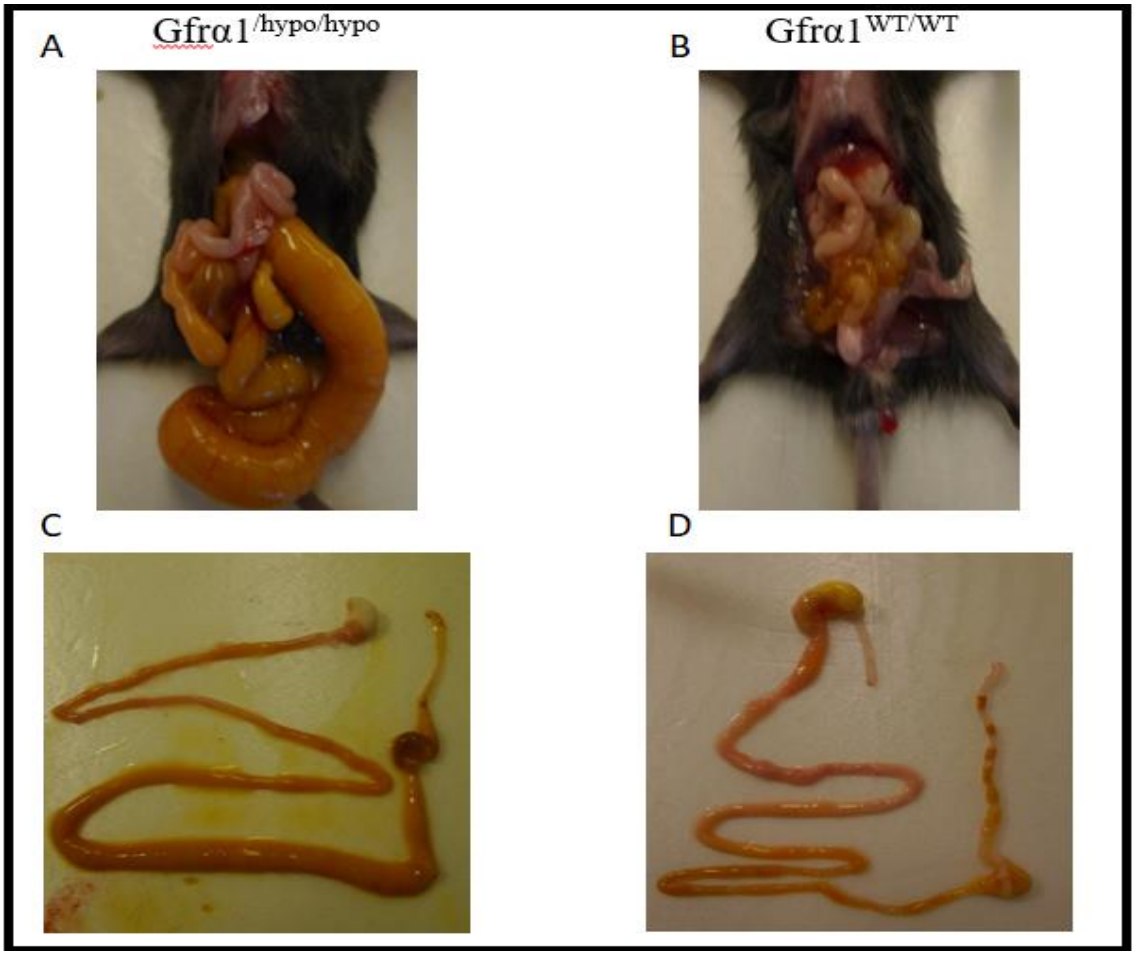
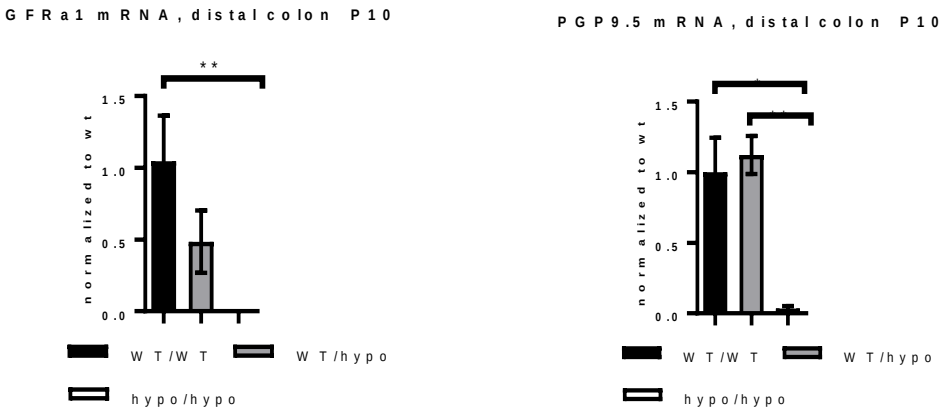


Figure 9. P16 *Gfra1*$^{hypo/hypo}$ vs. wild type GI tract. Aganglionosis in *Gfra1*$^{hypo/hypo}$ animals in the distal colon causes obstruction and does not allow passage of the stool. This results in gut distention up to the small intestine and thus works as a model of long segment Hirschsprung's disease. At the same time, the most distal part of the GI tract is empty because of intestinal obstruction (A, C). The wild type GI tract is not distended and there are stool pellets in the colon (D).

In the developing GI tract, *Gfra1* is expressed both by migrating enteric neural crest cells and in the mesenchyme (Durbec et al. 1996), whereas during the first postnatal week, Gfra1 expression is sustained only in ENCC derived cells. Because of reduced *Gfra1* expression, neural crest cells are unable to colonize the distal colon, and at

postnatal (P) day 10, no Gfra1 mRNA can be observed (Figure 10A). As expected from complete aganglionosis, levels of PGP9.5, a pan-neuronal marker, are also reduced to almost zero in the distal colon of *Gfra1*$^{hypo/hypo}$, (Figure 10B).



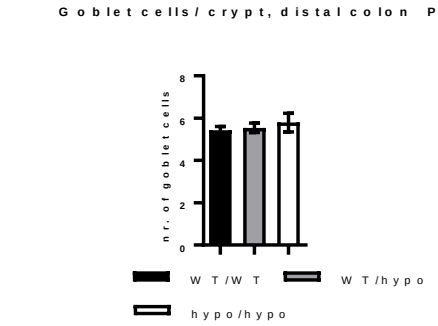
A **B**

Figure 10. Distal colon lacks enteric ganglia in *Gfra1*$^{hypo/hypo}$ mice. At P10 *Gfra1*$^{WT/hypo}$ have reduced *Gfra1* expression (A), but this has minimal effect on the ENS innervation as expression of pan-neuronal marker *PGP9.5* is comparable to wild types (B). In contrast, *Gfra1*$^{hypo/hypo}$ have very low levels of *PGP9.5* due to aganglionosis (B). Data is presented as mean ± SEM. *P*<0.05= *, *P*<0.01**, P<0.001=***. A n=5-7, B n=4-14. Statistical analyses were performed with one-way-ANOVA and Tukey's multiple comparison tests.

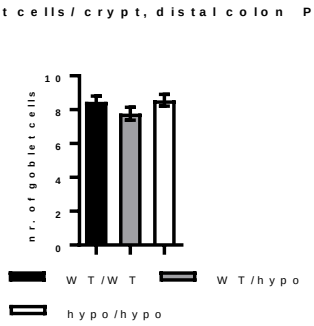
3.2 Goblet cells are altered in the colon of *Gfra1*$^{hypo/hypo}$ mice

Goblet cells are involved in intestinal homeostasis. In order to better understand the order of the events leading to the final stages of HAEC including sepsis, I counted goblet cells in distal colon crypts at P2, P5, and P10 and quantified the mucin profile.

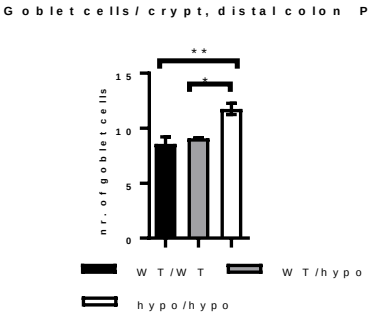
Goblet cell hyperplasia was observed at P10 in the GI tract of *Gfra1*$^{hypo/hypo}$ mice, but not earlier (Figure 11). On the other hand, *Gfra1*$^{WT/hypo}$ have same amount of goblet cells at every time point as wild type controls.



A



B

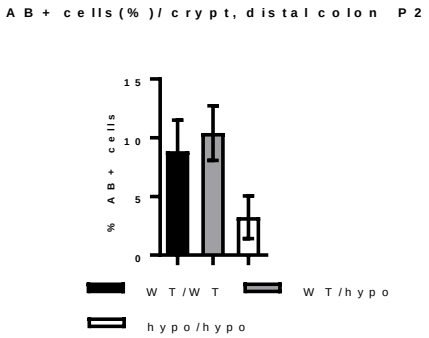


C

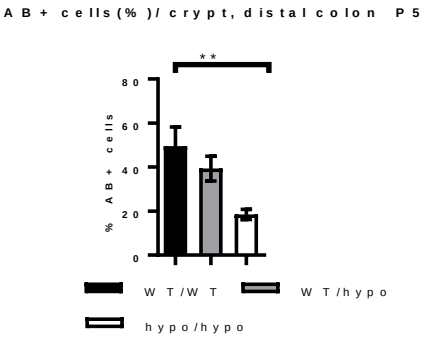
Figure 11. Number of goblet cells in the distal colon. At P2- and P5 no difference is observed (A, B). At P10, *Gfra1*$^{hypo/hypo}$ mice have over a 30% increase in number of goblet cells compared to wild types and heterozygotes. (C). Data is presented as mean ± SEM. *P*<0.05= *, *P*<0.01**, P<0.001=***. A n=3-8, B n=4-5, C n=3-5. Statistical analyses were performed with one-way-ANOVA and Tukey's multiple comparison tests

Next, I quantified the mucin profile inside goblet cells with AB/PAS staining. While only a nonsignificant trend towards a reduction of acidic mucins was observed at P2 in *Gfra1*$^{hypo/hypo}$ distal colon, this reduction was significant at P5 and this effect persisted also to P10 (Figure 12). Interestingly, *Gfra1*$^{WT/hypo}$ mice, which have an apparently normal ENS throughout the gut, also had a significant reduction in acidic mucin, however only at P10 (Figure 12C). This suggests that mucin synthesis may be controlled independent of the presence or absence of the ENS as a function of GFRa1 levels, thus warranting further investigation. As shown in Figure 13, a different mucin

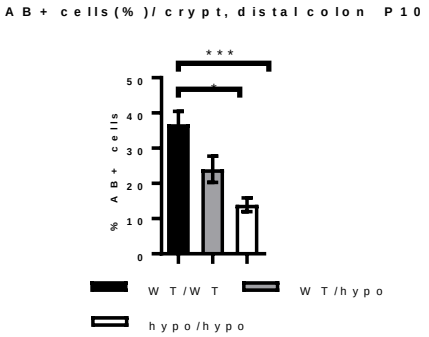
profile in *Gfra1*$^{hypo/hypo}$ colons is clearly visible relative to controls already at P5, showing that shift in mucin profile synthesis occurs before goblet cell hyperplasia.



A



B



C

Figure 12. Goblet cell mucin profile in the distal colon. No significant change in mucin profile is observed at P2, aside from a nonsignificant trend towards reduction in *Gfra1*$^{hypo/hypo}$ distal colon (A). A significant reduction in acidic mucin is seen from P5 onwards in *Gfra1*$^{hypo/hypo}$ distal colon (B, C). Interestingly, at P10, *Gfra1*$^{WT/hypo}$ have less acidic mucin in the goblet cells compared to wild types (C). Data is presented as mean ± SEM. *P*<0.05= *, *P*<0.01**, P<0.001=***. A n=3-4, B n=4-5, C n=4-6. Statistical analyses were performed with one-way-ANOVA and Tukey's multiple comparison tests.

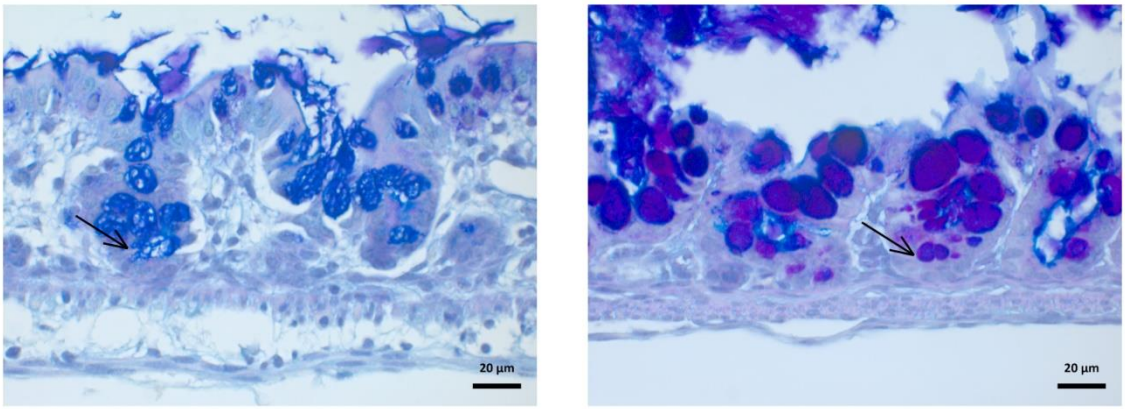


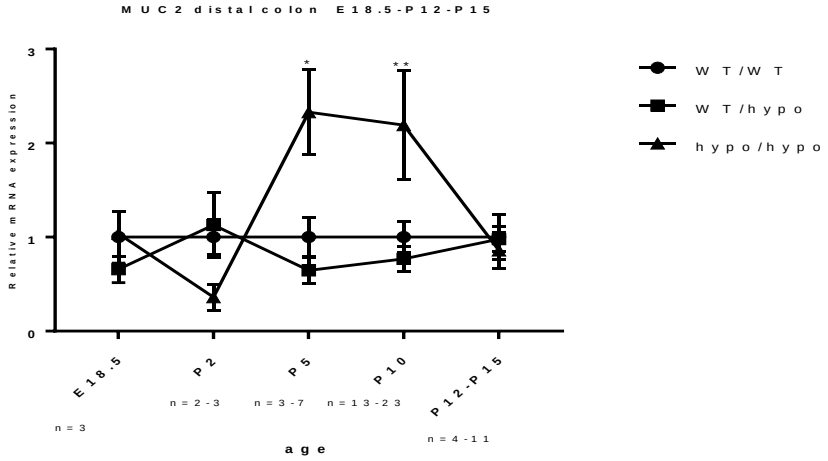
Figure 13. *Gfra1*$^{hypo/hypo}$ colon crypt vs wild type crypt at P5. At the bottom of the wild type crypt goblet cells produce more light-blue stained acidic mucin (black arrow, left panel). Mucins at the bottom of the *Gfra1*$^{hypo/hypo}$ crypt stain magenta/purple indicating a shift away from acidic mucin (black arrow, right panel). Samples were stained with AB-PAS staining. Scale is indicated in each image.

3.3 Goblet cell related gene expression in the distal colon

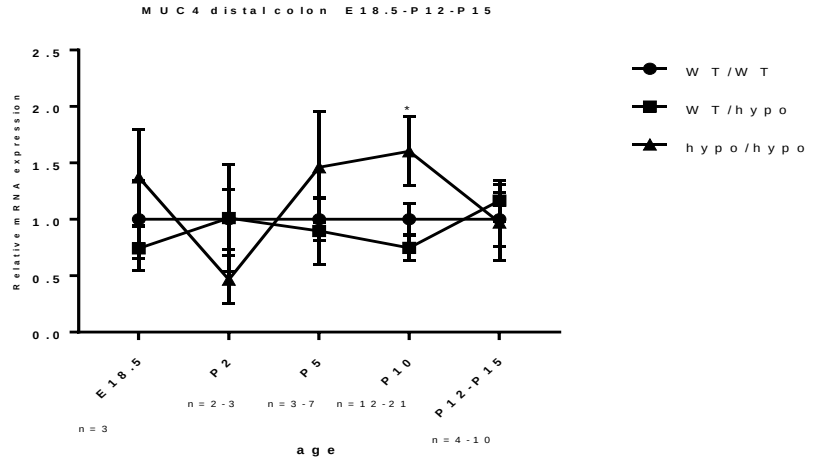
Several studies have shown that *MUC* transcripts or protein levels are altered in HSCR patients (Aslam et al. 1997a; Aslam et al. 1997b; Aslam et al. 1999; Thiagarajah et al. 2014; Nakamura et al. 2018). Thus, *Muc2* and *Muc4* mRNA levels were studied at five different timepoints (E18.5, P2, P5, P10 and P12-P15).

MUC2, the most abundant mucin in the gut, was upregulated in the distal colon of *Gfra1*$^{hypo/hypo}$ animals between P5 and P10, after which it returned to control levels at P12-P15 (Figure 14a).

Muc4 had similar expression levels as *Muc2*, but a statistical difference was only seen at P10 (Figure 14b).



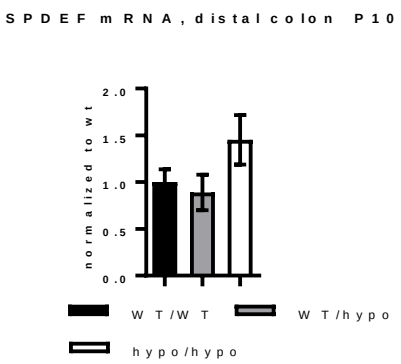
A



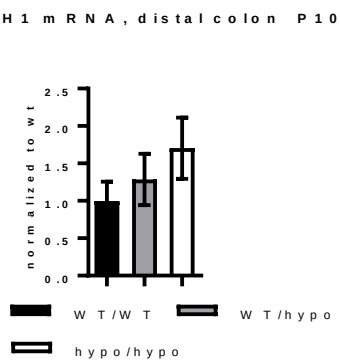
B

Figure 14. *Muc2* (A) and *Muc4* (B) expression in the distal colon between E18.5-P12-P15. Two-fold increase was observed in *Muc2* expression of *Gfra1*$^{hypo/hypo}$ animals at P5 and P10 (A). An increase in *Muc4* expression in *Gfra1*$^{hypo/hypo}$ animals was observed only at P10 (B). Data is presented as mean ± SEM. $P<0.05$= *, $P<0.01$**, $P<0.001$=***. Separate statistical analyses were performed at each timepoint (E18.5, P2, P5, P10, P12-P15) with one-way-ANOVA and Tukey's multiple comparison tests

Goblet cells differentiate from stem cells at the bottom of the crypt. For this to occur, transcription factors ATOH1 and SPDEF are required (Lo et al. 2017). At P10, there was a trend towards upregulation in *Gfra1*$^{hypo/hypo}$ animals in both of these genes, but it did not reach statistical significance (Figure 15).



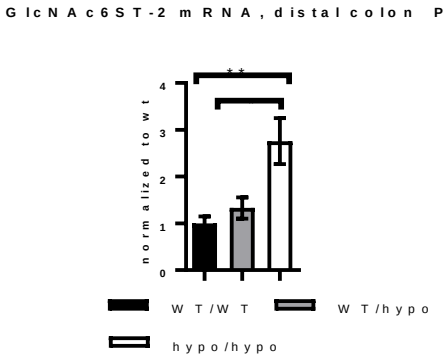
A



B

Figure 15. Expression of *Spdef* (A) and *Atoh1* (B) in the distal colon at P10. No statisical difference between groups. Data is presented as mean ± SEM. A n=5-7, B n=5-7. Statistical analyses were performed with one-way-ANOVA and Tukey's multiple comparison tests.

Mucins produced by goblet cells undergo extensive post-translational modifications, whereupon sugars and modified sugars are added to the protein backbone (As reviewed in Bergström and Xia 2013). N-acetylglucosamine 6-O-sulfotransferase-2 (GlcNAc6ST-2) has been shown to be crucial for the transfer of sulphate groups in the mucin protein backbone (Tobisawa et al. 2010). *Gfra1*$^{hypo/hypo}$ animals show a significant increase in *Glcnac6st-2* mRNA as compared to both wild types and heterozygous animals (Figure 16).



A

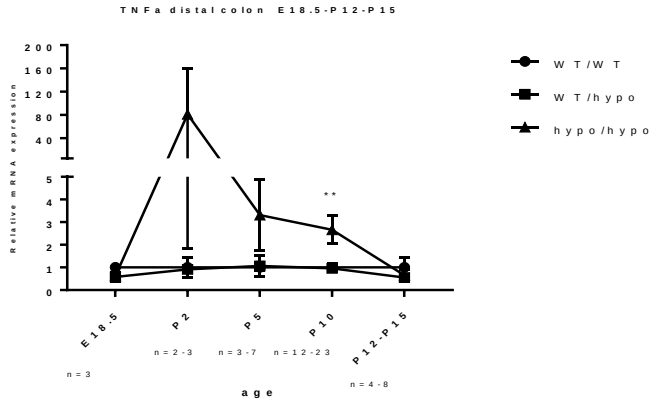
Figure 16. Expresion of *Glcna6st-2* in the distal colon at P10. Three-fold increase in *Glcnac6st-2* was observed in *Gfra1*$^{hypo/hypo}$ (A). Data is presented as mean ± SEM.

P<0.05= *, *P*<0.01**, P<0.001=***. A n=8-10, B n=7-9. Statistical analyses were performed with one-way-ANOVA and Tukey's multiple comparison tests.

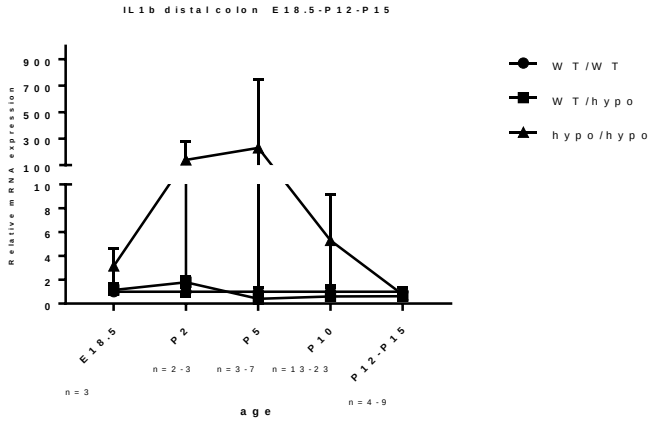
3.4 Inflammation genes

Inflammation in the gut has been reported in HSCR patients (Frykman et al 2015b; Demehri et al. 2016). To test for inflammation in our animals, the following cytokines were tested with qPCR: *Ifng*, *Il1b*, *Il1a*, *Il2*, *Il4*, *Il5*, *Il6*, *Il10*, *Il13*, *Il23*, *Tnfa*, *Tgfb1*. Out of all tested cytokines, *TNFa* mRNA was the only one that was significantly upregulated, and only at timepoint P10 in *Gfra1*$^{hypo/hypo}$ mice. There was already a trend at P2 onwards, but potentially due to a relatively low n there, was no statistical significance (Figure 17). *Il1b* had a similar expression pattern across genotypes to *Tnfa*, but due to high variation, there was also no significant difference (Figure 17b). For *Tgfb1* expression, no diffrence was seen at any point.

Il5, *Il10* and *Il23* were analyzed at P10 but no statisical difference was seen. Still, there was trend towards upregulation of *Il1a* (Figure 18).



A



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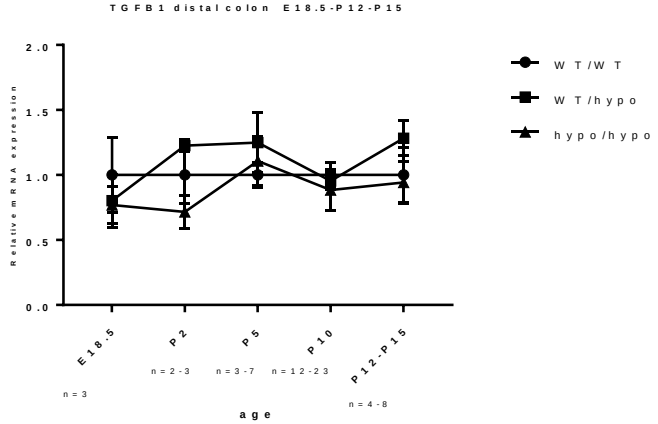
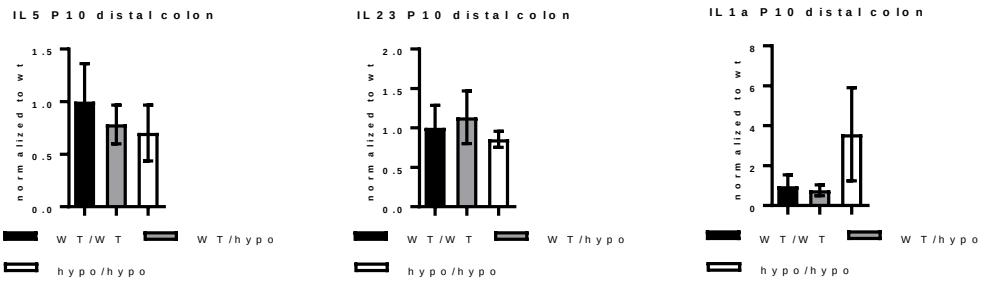


C

Figure 17. *Tnfa* (A), *Il1b* (B), *Tgfb1* (C) expression in the distal colon between E18.5-P12-P15. Only *Tnfa* was significantly increased at P10 in *Gfra1*$^{hypo/hypo}$ animals (A). Data is presented as mean ± SEM. $P<0.05$= *, $P<0.01$**, P<0.001=***. Statistical analyses were performed at every timepoint (E18.5, P2, P5, P10, P12-P15) with one-way-ANOVA and Tukey's multiple comparison tests.



A **B** **C**

Figure 18. *Il5* (A), *Il23* (B) and *Il1a* (C) expression in the distal colon at P10. No statisical difference between groups. Data is presented as mean ± SEM. A n=3-5, B n=5-7, C=4-7. Statistical analyses were performed with one-way-ANOVA and Tukey's multiple comparison tests.

Serum cytokine levels were also measured using ELISA. The following cytokines were tested: IFNg, IL1b, IL4, IL5, IL6, IL9, IL12, TNFa. Out of 8 tested cytokines, 3 gave signal: IL5, IL6 and TNFa. No differences were observed, although at P14-P16 group one *Gfra1*$^{hypo/hypo}$ mouse had elevated levels of TNFa and IL6 (Figure 19).

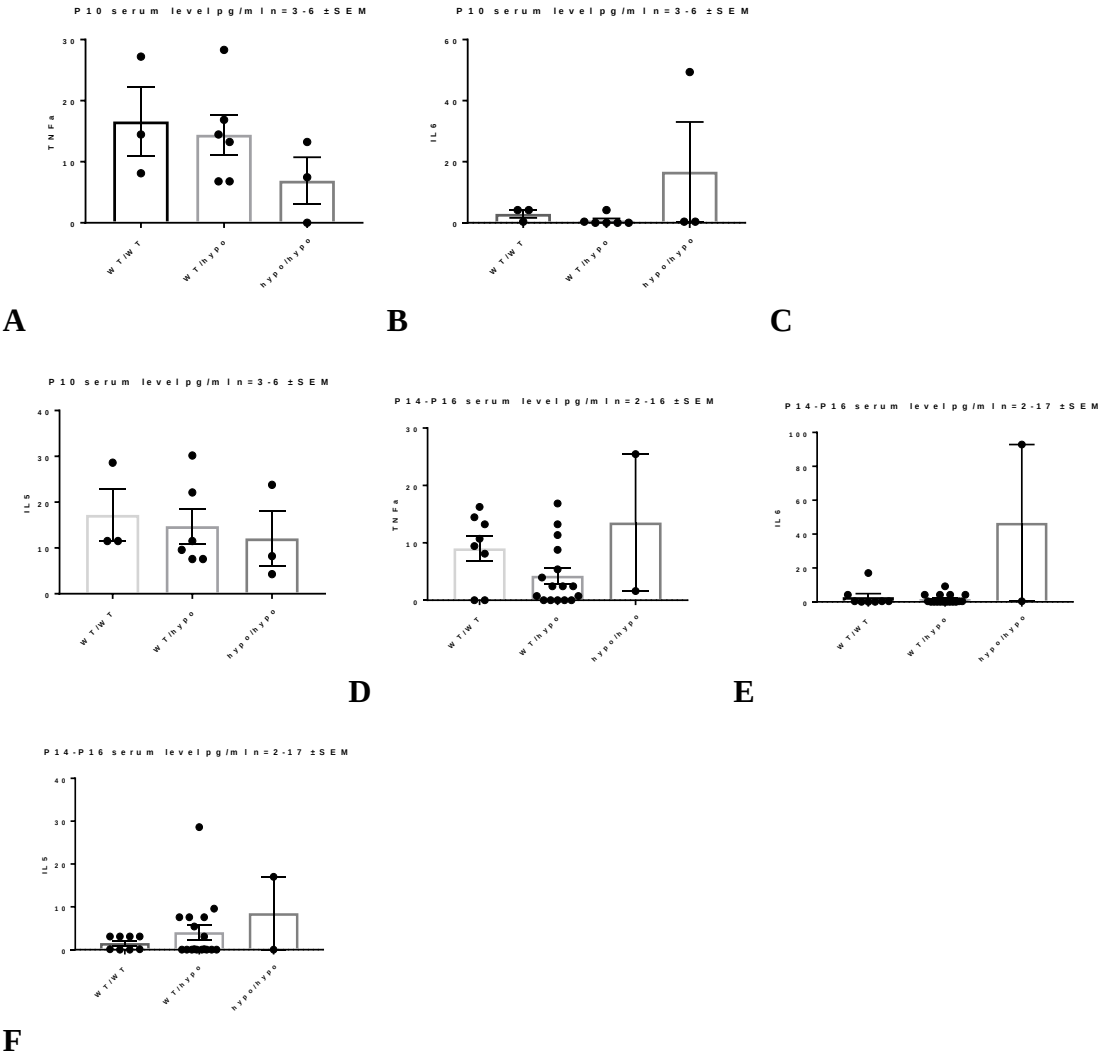


Figure 19. TNFa (A,D), IL6 (B,E) and IL5 (C,F) levels in the serum at P10-P14-P16. No differences were observed. At P14-P16 one *Gfra1*$^{hypo/hypo}$ had elevated level of TNFa and IL6 (D,E). Data is presented as mean ± SEM. Statistical analyses were performed with one-way-ANOVA and Tukey's multiple comparison tests.

3.5 Small intestine

The duodenum was also analyzed to see if changes in the goblet cells in the colon are due to reduced GDNF signalling or lack of the ENS. Histological images of the duodenum are shown in Figure 20.

Goblet cells were counted and analysed for mucin at P10 in the proximal duodenum. No difference in either parameter was seen (Figure 21a). Goblet cells in the villi were mainly PAS-positive (Figure 21b). Since the duodenum of *Gfra1*$^{hypo/hypo}$ mice is normally innervated and no changes in goblet cells were observed, changes in the colon can be attributed to a lack of innervation, rather than a lack of GDNF signalling.

Finally, cytokine mRNA levels were analyzed from the small intestine to see if the inflammation that was seen in the colon was also seen in duodenum (Figure 22). However, no changes in the expression of cytokines were observed.

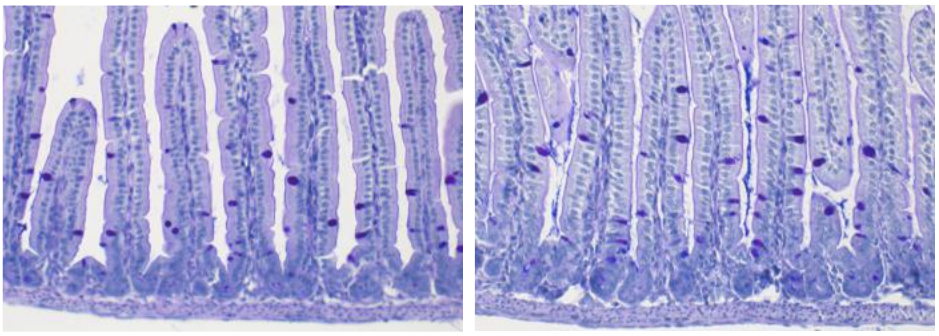
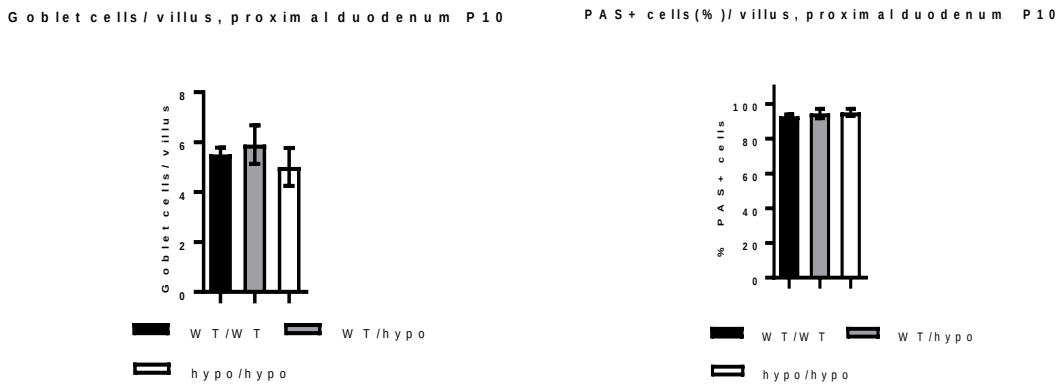


Figure 20. Wild type (left) vs *Gfra1*$^{hypo/hypo}$ (right) proximal duodenum at P10. Goblet cells in both duodena express mainly neutral mucins as visualized with AB-PAS staining.



A **B**

Figure 21. Goblet cells in the proximal duodenum at P10. No difference in the number of goblet cells or in their mucin composition was observed. Data is presented as mean ±

SEM. A n=4, B n=4. Statistical analyses were performed with one-way-ANOVA and Tukey's multiple comparison tests.

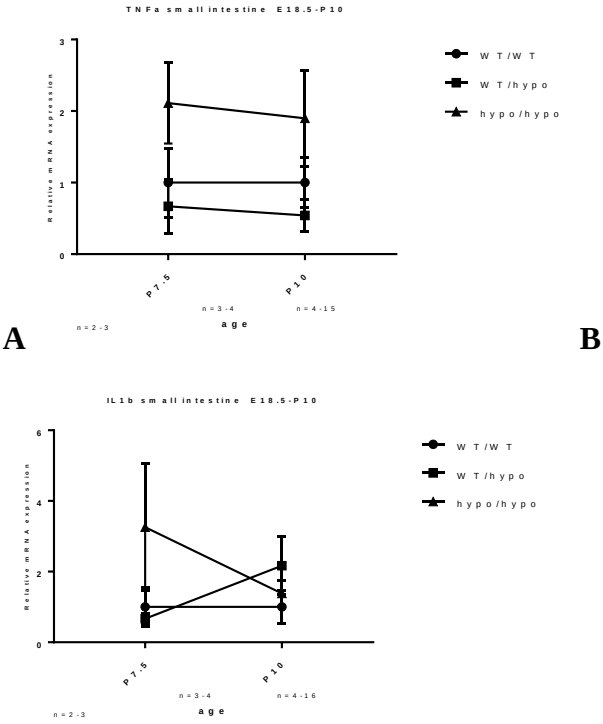


Figure 22. *Tnfa* (A) and *Il1b* (B) expression in the small intestine at P7.5 and P10. Data is presented as mean ± SEM. Statistical analyses were performed at each timepoint (P7.5, P10) with one-way-ANOVA and Tukey's multiple comparison tests.

4 DISCUSSION

The aim of this study was to provide insight into the order of events which contribute to HAEC. I investigated goblet cell mucin profile changes, goblet cell hyperplasia, epithelial histology, and analysed molecular parameters of goblet cell function as well as inflammation with QPCR in the gut and with ELISA in the serum. For this, GFRa1 hypomorphic mice were examined at various time points beginning from E18.5. No changes were observed in mRNA expression of mucins (*Muc2*, *Muc4*) or inflammatory

genes (*Il1b, Tgfb1, Tnfa*) at E18.5. At P2, goblet cell number in the crypts of the distal third of the colon was similar between groups, and there was no statistical difference in mucin profile, even though there seems to be a trend towards downregulation of AB+ mucin in hypomorphs, signalling a shift away from a more acidic mucin profile. QPCR-analysis at P2 revealed a trend in upregulation of early phase inflammatory genes *IL1b* and *Tnfa*, but perhaps due to a low number of animals, it did not reach statistical significance. P5 was first time point where statistically significant changes could be seen in *Gfra1*$^{hypo/hypo}$ animals. A trend towards upregulation of *Tnfa* was observed at this time point. Interestingly, *Muc2*, the main secreted mucin was upregulated at this point. This is in opposition to other studies, where either no change or downregulation of *Muc2* has been observed (Thiagarajah et al. 2014; Nakamura et al. 2018) A potential reason for this discrepancy might be that the *MUC2* mRNA was measured at later time points in previous studies when there are already secondary changes. Histological analyses showed mucin retention in the colon, which might reflect mucin overproduction indicated by upregulated *MUC2*. However, *MUC2* mRNA and protein levels do not always correlate, so a conclusive analysis would require measurement of protein levels of MUC2.

In addition, goblet cells in the distal colon at time point P5 had started to produce abnormal mucin in *Gfra1*$^{hypo/hypo}$ animals, observed at the bottom of the crypt. Normally, goblet cells at the bottom of the crypts of the distal colon produce acidic mucin, shown by AB+ staining. In *Gfra1*$^{hypo/hypo}$ mice, there was a shift towards neutral mucin production, shown by an increase in PAS+ staining. It is not known why this shift in mucin profile occurs. However, studies have implied that acidic mucin has protective properties and may prevent bacterial adherence into the epithelium (Tobisawa et al. 2012). Sulfation of mucins is one of the main ways to make mucins acidic. In this process, an acidic sulphate group is added to the sugar chain of mucin proteins.

Interestingly at P10, the *Gfra1*$^{WT/hypo}$ mice also had less acidic mucin. These mice have a normal lifespan and are fertile, with seemingly no gut related issues. Further study is required to see if these mice are more susceptible to DSS, since they have less protective acidic mucin.

In a study done by Tobisawa et al., it was shown that mice that were deficient in N-acetylglucosamine 6-O-sulfotransferase-2 (GlcNAc6ST-2), present an absence of acidic mucin (AB+). These mice were more susceptible to dextran sodium sulphate (DSS) induced colitis. For this reason, *Glcnac6st-2* mRNA expression was analysed at P5 where a shift away from acidic mucin is seen for the first time in *Gfra1*$^{hypo/hypo}$ mice, and at P10 where the same effect persisted. No change at P5 was observed (data not shown), but at P10, *Glcnac6st-2* was significantly upregulated. If *Glcnac6st-2* correlates with acidic mucin production, the observation that *Glcnac6st-2* mRNA is upregulated while the amount of acidic mucin is diminished requires further study. It is possible that mRNA levels do not reflect the protein levels. Also, the interpretation of *Glcnac6st-2* mRNA is complicated by the fact that *Glcnac6st-2* is also expressed in high endothelial venules (HEVs) (Bistrup et al. 1999). Thus, immunohistological analysis is required to address if protein derived from the *Glcnac6st-2* allele is changed, and if so, in which cells. As one option, upregulation of *Glcnac6st-2* expression may reflect an attempt to compensate for the decline of acidic mucin production around P5 and P10.

At P10, goblet cell hyperplasia is detected in *Gfra1*$^{hypo/hypo}$ mice. qPCR-analysis did not show a significant upregulation of *Atoh1* and *Spdef*, transcription factors involved in goblet cell differentiation. This is seemingly in opposition to the upregulation observed in *Ednrb* null mice (Thiagarajah et al. 2014). That said, we did observe around 30% goblet cell hyperplasia in our *Gfra1*$^{hypo/hypo}$ animals, and there was a trend in *Atoh1* and *Spdef* upregulation (around 1.5 fold). It is possible that statistical significance would have been reached if the number of mice analyzed had been higher.

ELISA results indicated that systemic inflammation did not occur P10-P14-P16 although one *Gfra1*$^{hypo/hypo}$ animal had high levels of IL6 and TNFa at P14-16. This indicates that inflammation is neither an early nor mid-stage event in HAEC. Moreover, inflammation markers were not upregulated in the small intestine, indicating that at the mRNA level only the aganglionic colon segment is affected.

One of the key findings of this study is that changes in goblet cells seem to be due to a lack of the ENS rather than reduced GDNF signalling directly, as no changes were observed in the small intestine. There the ENS has developed normally, yet there is reduction *Gfra1* expression. This indicates that the changes observed in the colon are

likely due to the lack of ENS innervation, rather than a direct effect from GDNF-GFRa1-RET signalling itself.

5 CONCLUSION

The GFRa1 hypomorphic mouse line is the first postnatally viable HSCR model that affects GDNF-GFRα1-RET signalling. We have observed a shift away from acidic mucin production, followed by goblet cell hyperplasia, and an increase in certain cytokine production in our *Gfra1*$^{hypo/hypo}$ mice. There was already a trend in the production of certain cytokines at P2, with no significance achieved until P10, while a shift in mucin profile, missing at P2, already appeared at P5. Nevertheless, it is likely that these events happen relatively concurrently, likely between P2-P5. Analysis at P3-P4 would be required to see if these events indeed coincide or if one precedes the other. Still, what drives goblet cells to begin producing less acidic mucin is not known but constitutes an important future research question. It is entirely possible that the lack of an ENS causes disruption in the epithelial-immune-ENS axis, which alters the goblet cell function. In conclusion, we have found that GFRa1 hypomorphic animals develop a HSCR-like phenotype where altered mucin production by goblet cells is the first histopathological hallmark preceding HAEC. Possibly, pharmacological prevention of this mucin shift may reduce or abolish HAEC, but whether this is the case requires further studies. Key histopathological findings summarizing this work as well as the work by other group members are shown in Figure 23.

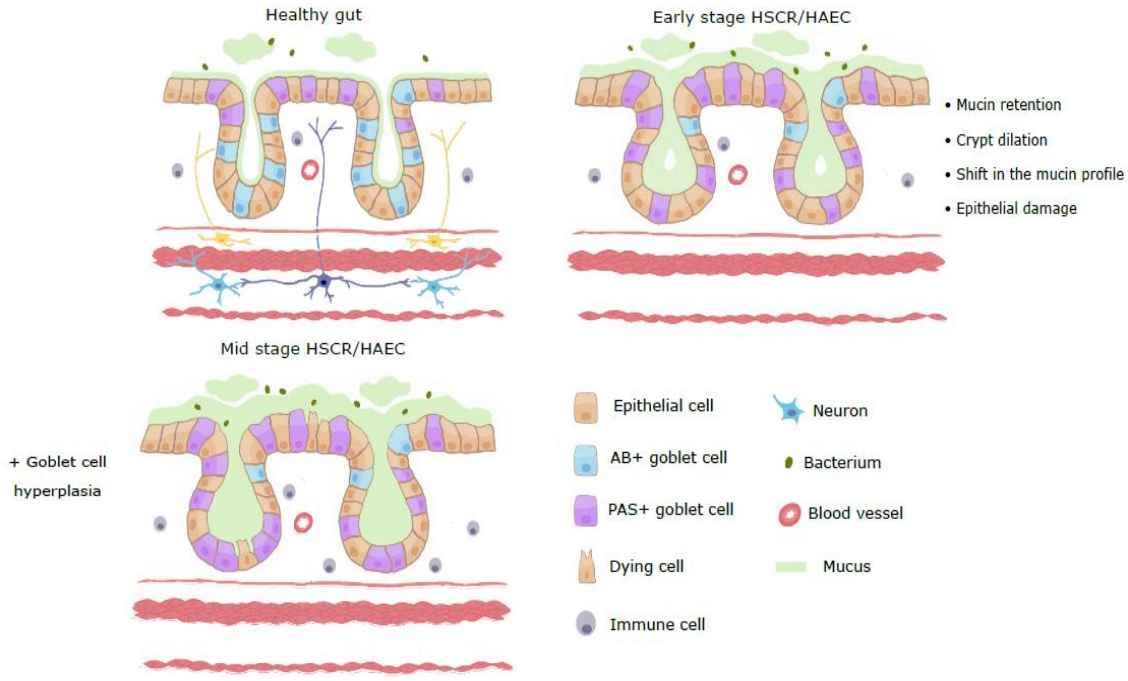


Figure 23. Graphical illustration of key histopathlogical findings in *Gfra1*$^{hypo/hypo}$ mice.

6 ACKNOWLEDGMENTS

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7 REFERENCE

Ahn DH, Crawley SC, Hokari R, Kato S, Yang SC, Li JD, Kim YS: TNF-alpha activates MUC2 transcription via NF-kappaB but inhibits via JNK activation. Cell Physiol Biochem., 15(1-4): 29-40, 2005

Airaksinen MS and Saarma M: The GDNF family: Signalling, biological functions and therapeutic value. Nat Rev Neurosci, 3(5): 383-394, 2002

Anderson RB, Newgreen DF, Young HM: Neural crest and the development of the enteric nervous system. Adv Exp Med Biol, 589: 81-196, 2006a

Anderson RB, Turner KN, Nikonenko AG, Hemperly J, Schachner M, Young HM: The cell adhesion molecule l1 is required for chain migration of neural crest cells in the developing mouse gut. Gastroenterology, 130(4): 1221-1232, 2006b

Aslam A, Spicer RD, Corfield AP: Biochemical analysis of colonic mucin glycoproteins in children with Hirschsprung disease show disease specific alterations. Biochem Soc Trans, 25(1): 8S, 1997a

Aslam A, Spicer RD, Corfield AP: Children with Hirschsprung's disease have an abnormal colonic mucus defensive barrier independent of the bowel innervation status. J Pediatr Surg, 32(8): 1206-1210, 1997b

Aslam A, Spicer RD, Corfield AP: Turnover of radioactive mucin precursors in the colon of patients with hirschsprung's disease correlates with the development of enterocolitis. J Pediatr Surg, 33(1): 103-105, 1998

Aslam A, Spicer RD, Corfield AP: Histochemical and genetic analysis of colonic mucin glycoproteins in Hirschsprung's disease. J Pediatr Surg, 34(2): 330-333, 1999

Attié T1, Pelet A, Edery P, Eng C, Mulligan LM, Amiel J, Boutrand L, Beldjord C, Nihoul-Fékété C, Munnich A, Ponder BAJ and Lyonnet: Diversity of RET proto-oncogene mutations in familial and sporadic Hirschsprung disease. Hum Mol Genet, 4(8): 1381-6, 1995

Austin KM: The pathogenesis of hirschsprung's disease-associated enterocolitis. Semin Pediatr Surg, 21(4): 319-327, 2012

Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI: Host-bacterial mutualism in the human intestine. Science (New York, N.Y.), 307(5717): 1915-1920, 2005

Barlow A, de Graaff E, Pachnis V: Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. Neuron, 40(5): 905-916, 2003

Bayliss WM and Starling EH: The movements and innervation of the small intestine. J Physiol, 24(2): 99–143, 1899

Belkaid Y, Bouladoux N, Hand TW: Effector and memory T cell responses to commensal bacteria. Trends in Immunol, 34(6), 299-306, 2013

Bergstrom KS and Xia L: Mucin-type O-glycans and their roles in intestinal homeostasis. Glycobiology, 23(9): 1026-1037, 2013

Bistrup A, Bhakta S, Lee JK, Belov YY, Gunn MD, Zuo FR, . . . Hemmerich S: Sulfotransferases of two specificities function in the reconstitution of high endothelial cell ligands for L-selectin. J Cell Biol, 145(4): 899-910, 1999

Boyd JG and Gordon T: Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. Mol Neurobiol, 27(3):277-324, 2003

Britsch S, Goerich DE, Riethmacher D, Peirano RI, Rossner M, Nave KA, Birchmeier C, Wegner M: The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev. 15(1): 66–78, 2001

Bronner ME: Formation and migration of neural crest cells in the vertebrate embryo. Histochem Cell Biol, 138(2): 179-186, 2012

Burns AJ and Douarin NM: The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. Development, 125(21): 4335-47, 1998

Cani PD: Human gut microbiome: Hopes, threats and promises. Gut, 67(9): 1716-1725, 2018

Cheng Z, Zhao L, Dhall D, Ruegger PM, Borneman J, Frykman PK: Bacterial microbiome dynamics in post pull-through hirschsprung-associated enterocolitis (HAEC): An experimental study employing the endothelin receptor B-null mouse model. Front Surg, 5:30; 2018

Cohen M, Varki NM, Jankowski MD, Gagneux P: Using Unfixed, Frozen Tissues to Study Natural Mucin Distribution. J Vis Exp, (67): 3928, 2002

Demehri FR, Frykman PK, Cheng Z, Ruan C, Wester T, Nordenskjöld A, Kawaguchi A, Hui TT, Granström AL, Funari V, Teitelbaum DH: Altered fecal short chain fatty acid composition in children with a history of Hirschsprung-associated enterocolitis. J Pediatr Surg. 51(1): 81–86, 2016

Druckenbrod NR and Epstein ML: Age-dependent changes in the gut environment restrict the invasion of the hindgut by enteric neural progenitors. Development, 136(18): 3195-3203, 2009

Durbec PL, Larsson-Blomberg LB, Schuchardt A, Costantini F, Pachnis V: Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. Development, 122(1): 349-358, 1996

Emison ES, McCallion AS, Kashuk, CS, Bush RT, Grice E, Lin S, Chakravarti A: A common sex-dependent mutation in a RET enhancer underlies hirschsprung disease risk. Nature, 434(7035): 857-863, 2005

Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, Johnson EM Jr, Milbrandt J: GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron, 21(2): 317-324, 1998

Fagarasan S and Honjo T: Intestinal IgA synthesis: Regulation of front-line body defences. Nat Rev.Immunol, 3(1): 63-72, 2003

Focke PJ, Schiltz CA, Jones SE, Watters JJ, Epstein ML: Enteric neuroblasts require the phosphatidylinositol 3-kinase pathway for GDNF-stimulated proliferation. J Neurobiol, 47(4): 306-317, 2001

Frykman PK, Cheng Z, Wang X, Dhall D: Enterocolitis causes profound lymphoid depletion in endothelin receptor B- and endothelin 3-null mouse models of hirschsprung-associated enterocolitis. Eur J Immunol, 45(3): 807-817, 2015a

Frykman PK, Nordenskjöld A, Kawaguchi A, Hui TT, Granström AL, Cheng Z, Tang J, Underhill DM, Iliev I, Funari VA, Wester T; HAEC Collaborative Research Group (HCRG): Characterization of Bacterial and Fungal Microbiome in Children with Hirschsprung Disease with and without a History of Enterocolitis: A Multicenter Study. PLoS One, 24;10(4), 2015b

Frykman PK and Short SS: Hirschsprung-associated enterocolitis: Prevention and therapy. Semin Pediatr Surg, 21(4): 328-335, 2012

Furness JB, Callaghan BP, Rivera LR, Cho HJ: The enteric nervous system and gastrointestinal innervation: Integrated local and central control. Adv Exp Med Biol, 817: 39-71, 2014

Gershon MD and Tack J: The serotonin signaling system: From basic understanding to drug development for functional GI disorders. Gastroenterology, 132(1): 397-414, 2007

Gianino S, Grider JR, Cresswell J, Enomoto H, Heuckeroth RO: GDNF availability determines enteric neuron number by controlling precursor proliferation. Development, 130(10): 2187-2198, 2003

Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, . . . Nelson KE: Metagenomic analysis of the human distal gut microbiome. Science, 312(5778): 1355-1359, 2006

Gosain A: Established and emerging concepts in Hirschsprung's-associated enterocolitis. Pediatr Surg Int, 32(4): 313-20, 2016

Guo F, Debidda M, Yang L, Williams DA, Zheng Y: Genetic deletion of Rac1 GTPase reveals its critical role in actin stress fiber formation and focal adhesion complex assembly. J Biol Chem , 281(27): 18652-18659, 2006

Hansen MB: Neurohumoral control of gastrointestinal motility. Physiol Res , 52(1): 1-30, 2003

Hao MM and Young HM: Development of enteric neuron diversity. J Cell Mol Med, 13(7): 1193-1210, 2009

Hearn CJ, Murphy M, Newgreen D: GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. Dev Biol, 197(1): 93-105, 1998

Herbarth B, Pingault V, Bondurand N, Kuhlbrodt K, Hermans-Borgmeyer I, Puliti A, . . . Wegner M: Mutation of the sry-related Sox10 gene in dominant megacolon, a mouse model for human hirschsprung disease. Proc Natl Acad Sci U S A , 95(9): 5161-5165, 1998

Heuckeroth RO: Hirschsprung disease - integrating basic science and clinical medicine to improve outcomes. Nat Rev Gastroenterol Hepatol , 15(3): 152-167, 2018

Ibiza S, Garcia-Cassani B, Ribeiro H, Carvalho T, Almeida L, Marques R, . . . Veiga-Fernandes H: Glial-cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. Nature, 535(7612): 440-443, 2016

Imamura A, Puri P, O'Briain DS, Reen DJ: Mucosal immune defence mechanisms in enterocolitis complicating hirschsprung's disease. Gut, 33(6): 801-806, 1992

Johansson ME and Hansson GC: Immunological aspects of intestinal mucus and mucins. Nat Rev.Immunol, 16(10): 639-649, 2016

Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L and Hansson GC: The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A, 105(39): 15064-9, 2008

Kenny SE, Tam PK, Garcia-Barcelo M: Hirschsprung's disease. Semin Pediatr Surg, 19(3): 194-200, 2010

Khan WI and Collins SM: Immune-mediated alteration in gut physiology and its role in host defence in nematode infection. Parasite Immunol, 26(8-9): 319-26, 2004

Kiela PR and Ghishan FK: Physiology of intestinal absorption and secretion. Best Pract Res Clin Gastroenterol. , 30(2): 145-159, 2016

Kim J, Lo L, Dormand E and Anderson DJ: SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. Neuron, 38: 17–31, 20032018

Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M: Sox10, a novel transcriptional modulator in glial cells. J Neurosci, 18(1): 237-250, 1998

Lake JI and Heuckeroth RO: Enteric nervous system development: Migration, differentiation, and disease. Am J Physiol Gastrointest Liver Physiol , 305(1): G1-24, 2013

Lane PW and Liu HM: Association of megacolon with a new dominant spotting gene (dom) in the mouse. J Hered, 75(6): 435-439, 1984

Le Douarin NM and Teillet MA: The migration of neural crest cells to the wall of the digestive tract in avian embryo. J Embryol Exp Morphol., 30(1): 31-48, 1973

Lo YH, Chung E, Li Z, Wan YW, Mahe MM, Chen MS, . . . Shroyer NF: Transcriptional regulation by ATOH1 and its target SPDEF in the intestine. Cell Mol Gastroenterol Hepatol , 3(1): 51-71, 2016

Lui VC, Samy ET, Sham MH, Mulligan LM and Tam PK: Glial cell line-derived neurotrophic factor family receptors are abnormally expressed in aganglionic bowel of a subpopulation of patients with Hirschsprung's disease. Lab Invest, 82(6): 703-12, 2002

Margolis KG, Gershon MD, Bogunovic M: Cellular organization of neuroimmune interactions in the gastrointestinal tract. Trends in Immunol, 37(7): 487-501, 2016

Marty TL, Seo T, Matlak ME, Sullivan JJ, Black RE, Johnson DG: Gastrointestinal function after surgical correction of Hirschsprung's disease: long-term follow-up in 135 patients. J Pediatr Surg, 30(5): 655-8, 1995

Mattar AF, Coran AG, Teitelbaum DH: MUC-2 mucin production in hirschsprung's disease: Possible association with enterocolitis development. J Pediatr Surg , 38(3): 417-21; discussion 417-21, 2003

McConalogue K and Furness JB: Gastrointestinal neurotransmitters. Baillieres Clin Endocrinol Metab , 8(1): 51-76, 1994

Miyahara K, Kato Y, Seki T, Arakawa A, Lane GJ, Yamataka A: Neuronal immaturity in normoganglionic colon from cases of hirschsprung disease, anorectal malformation, and idiopathic constipation. J Pediatr Surg , 44(12): 2364-2368, 2009

Mowat AM and Agace WW: Regional specialization within the intestinal immune system. Nat Rev.Immunol, 14(10): 667-685, 2014

Murphy F and Puri P: New insights into the pathogenesis of hirschsprung's associated enterocolitis. Pediatr Surg Int , 21(10): 773-779, 2005

Mwizerwa O, Das P, Nagy N, Akbareian SE, Mably JD, Goldstein AM: Gdnf is mitogenic, neurotrophic, and chemoattractive to enteric neural crest cells in the embryonic colon. Dev Dyn, 240(6): 1402-1411, 2011

Nagy N and Goldstein AM: Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. Dev Biol, 293(1): 203-217, 2006

Nagy N and Goldstein AM: Enteric nervous system development: A crest cell's journey from neural tube to colon. Semin Cell Dev Biol , 66: 94-106, 2017

Nakamura H, Tomuschat C, Coyle D, O'Donnel AM, Lim T, Puri P: Altered goblet cell function in hirschsprung's disease. Pediatr Surg Int, 34(2): 121-128, 2018

Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E: Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development, 129(22): 5151-5160, 2002

Nijenhuis CM, Horst PG, Berg LT, Wilffert B: Disturbed development of the enteric nervous system after in utero exposure of selective serotonin re-uptake inhibitors and tricyclic antidepressants. part 1: Literature review. Br J Clin Pharmacol , 73(1): 16-26, 2012

Oeser K, Schwartz C, Voehringer D: Conditional IL-4/IL-13-deficient mice reveal a critical role of innate immune cells for protective immunity against gastrointestinal helminths. Mucosal Immunol, 8(3):672-82, 2015

Pachnis V, Mankoo B, Costantini: Expression of the c-ret proto-oncogene during mouse embryogenesis. Development, 119(4): 1005-1017, 1993

Paratore C, Goerich DE, Suter U, Wegner M and Sommer L: Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development, 128: 3949–3961, 2001

Parisi MA: Hirschsprung disease overview. In M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens & A. Amemiya (Eds.), GeneReviews((R)), 1993

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF: The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature, 399(6734): 366-370, 1999

Peters-van der Sanden MJ, Kirby ML, Gittenberger-de Groot A, Tibboel D, Mulder MP and Meijers C: Ablation of various regions within the avian vagal neural crest has differential effects on ganglion formation in the fore-, mid- and hindgut. Dev Dyn, 196(3): 183-94, 1993

Pierre JF, Barlow-Anacker AJ, Erickson CS, Heneghan AF, Leverson GE, Dowd SE, Gosain A: Intestinal dysbiosis and bacterial enteroinvasion in a murine model of hirschsprung's disease. J Pediatr Surg, 49(8): 1242-1251, 2014

Pini Prato A, Rossi V, Avanzini S, Mattioli G, Disma, N, Jasonni, V: Hirschsprung's disease: What about mortality? Pediatr Surg Int, 27(5): 473-478, 2011

Polley, TZ Jr, Coran AG, Wesley JR: A ten-year experience with ninety-two cases of hirschsprung's disease. including sixty-seven consecutive endorectal pull-through procedures. Ann Surg , 202(3): 349-355, 1985

Porokuokka LL, Virtanen HT, Lindén J, Sidorova Y, Danilova T, Lindahl M, Saarma M, Andressoo JO: Gfra1 Underexpression Causes Hirschsprung’s Disease and Associated Enterocolitis in Mice. Cell Mol Gastroenterol Hepatol , In press, 2018

Rao M and Gershon MD: The bowel and beyond: the enteric nervous system in neurological disorders. Nat Rev Gastroenterol Hepatol , 13(9): 517–528, 2016

Rao M and Gershon MD: Enteric nervous system development: What could possibly go wrong? Nat Rev.Neurosci, 19(9): 552-565, 2018

Rhodes JM, Black RR, Gallimore R, Savage A: Histochemical demonstration of desialation and desulphation of normal and inflammatory bowel disease rectal mucus by faecal extracts. Gut, 26(12): 1312-1318, 1985

Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, Barbacid M: Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature, 382(6586): 70-73, 1996

Schill EM, Lake JI, Tusheva OA, Nagy N, Bery SK, Foster L, . . . Heuckeroth RO: Ibuprofen slows migration and inhibits bowel colonization by enteric nervous system precursors in zebrafish, chick and mouse. Dev Biol, 409(2): 473-488, 2016

Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V: Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor ret. Nature, 367(6461): 380-383, 1994

Schütte A, Ermund A, Becker-Pauly C, Johansson ME, Rodriguez-Pineiro AM, Bäckhed F, Müller S, Lottaz D, Bond JS and Hansson GC: Microbial-induced meprin β cleavage in MUC2 mucin and a functional CFTR channel are required to release anchored small intestinal mucus. Proc Natl Acad Sci U S A, 111(34): 12396-401, 2014

Sender R, Fuchs S, Milo R: Revised estimates for the number of human and bacteria cells in the body. PLoS Biology, 14(8): 2016

Sharkey KA and Savidge TC2014: Role of enteric neurotransmission in host defense and protection of the gastrointestinal tract. Auton Neurosci, 181: 94-106, 2014

Southard-Smith EM, Kos L, Pavan WJ: Sox10 mutation disrupts neural crest development in dom hirschsprung mouse model. Nat Genet, 18(1): 60-64, 1998

Spencer N, Walsh M, Smith TK: Does the guinea-pig ileum obey the 'law of the intestine'? J Physiol, 517: 889-898, 1999

Srinivasan S, Anitha M, Mwangi S, Heuckeroth RO: Enteric neuroblasts require the phosphatidylinositol 3-kinase/akt/forkhead pathway for GDNF-stimulated survival. Mol Cell Neurosci , 29(1): 107-119, 2005

Stewart DR and von Allmen D: The genetics of hirschsprung disease. Gastroenterol Clin North Am , 32(3): 819-37, 2003

Taraviras S, Marcos-Gutierrez CV, Durbec P, Jani H, Grigoriou M, Sukumaran M, . . . Pachnis V: Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. Development, 126(12): 2785-2797, 1999

Thiagarajah JR, Yildiz H, CarlsonT, Thomas AR, Steiger C, Pieretti A, Goldstein AM: Altered goblet cell differentiation and surface mucus properties in hirschsprung disease. PloS One, 9(6): 2014

Tobisawa Y, Imai Y, Fukuda M, Kawashima H: Sulfation of colonic mucins by N-acetylglucosamine 6-O-sulfotransferase-2 and its protective function in experimental colitis in mice. J Biol Chem, 285(9): 6750-6760, 2010

Uesaka T, Nagashimada M, Enomoto H: GDNF signaling levels control migration and neuronal differentiation of enteric ganglion precursors. J Neurosci, 33(41): 16372-16382, 2013

Wendelschafer-Crabb G, Neppalli V, Jessurun J, Hodges J, Vance K, Saltzman D, . . . Kennedy WR: Mucosal nerve deficiency in chronic childhood constipation: A postmigration defect? J Ped Surg, 44(4): 773-782, 2009

Wu JJ, Chen JX, Rothman TP, Gershon MD: Inhibition of in vitro enteric neuronal development by endothelin-3: Mediation by endothelin B receptors. Development, 126(6): 1161-1173, 1999

Yntema CL and Hammond WS: The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J Comp Neurolol, 101(2): 515-541, 1954

Yoo BB and Mazmanian SK: The enteric network: Interactions between the immune and nervous systems of the gut. Immunity, 46(6): 910-926, 2017

Young HM, Bergner AJ, Anderson RB, Enomoto H, Milbrandt J, Newgreen DF, Whitington PM: Dynamics of neural crest-derived cell migration in the embryonic mouse gut. Dev Biol, 270(2): 455-473, 2004

Young HM and Newgreen D: Enteric neural crest-derived cells: Origin, identification, migration, and differentiation. Anat Rec., 262(1): 1-15, 2001

Zhao L, Dhall D, Cheng Z, Wang HL, Doherty TM, Bresee C, Frykman PK: Murine model of hirschsprung-associated enterocolitis II: Surgical correction of aganglionosis does not eliminate enterocolitis. J Ped Surg, 45(1): 206-11, 2010